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Journal of the American Association of Clinical Chemists

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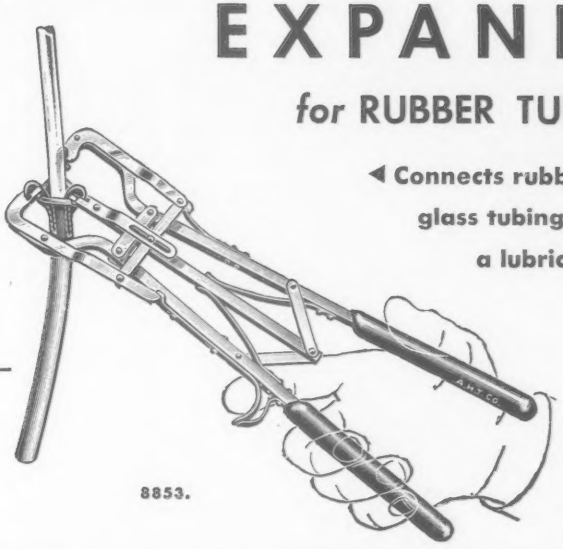

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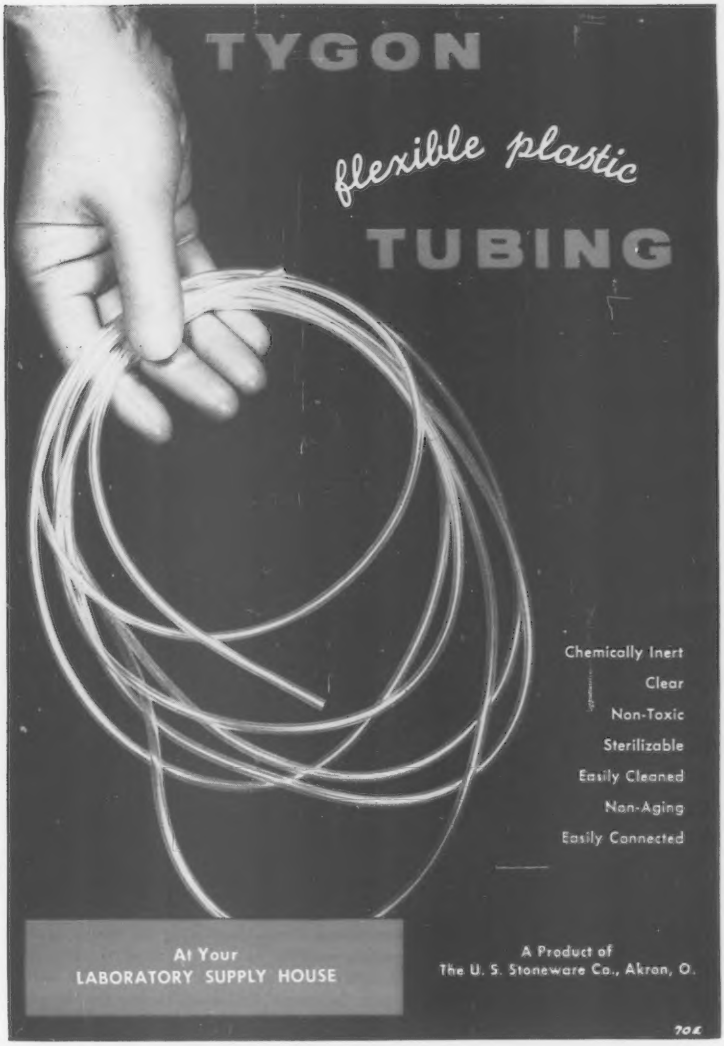
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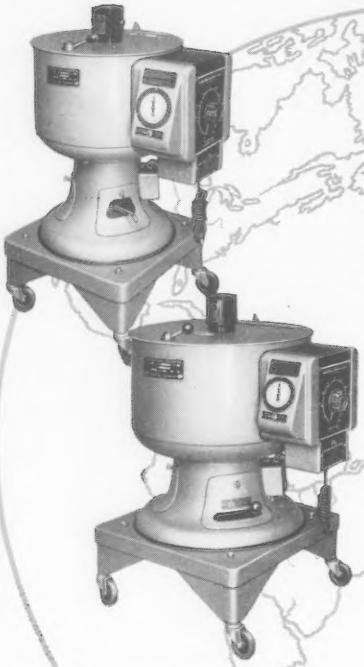

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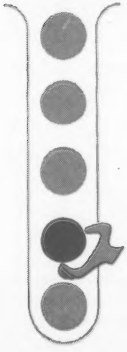

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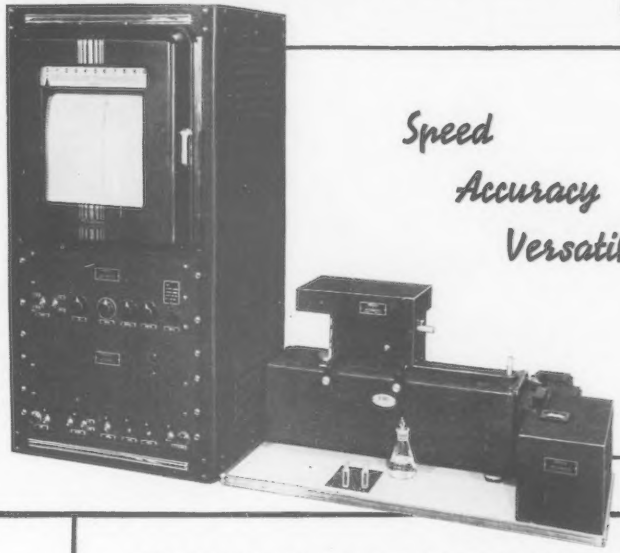

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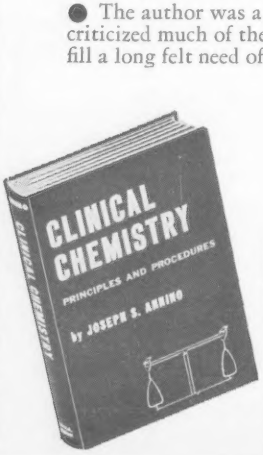

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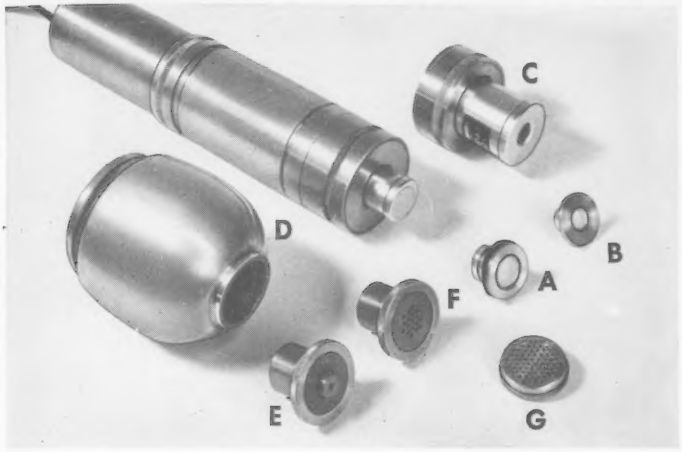

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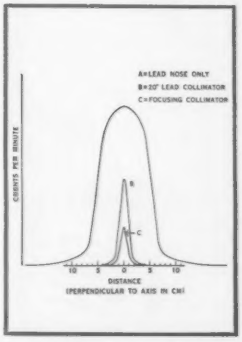

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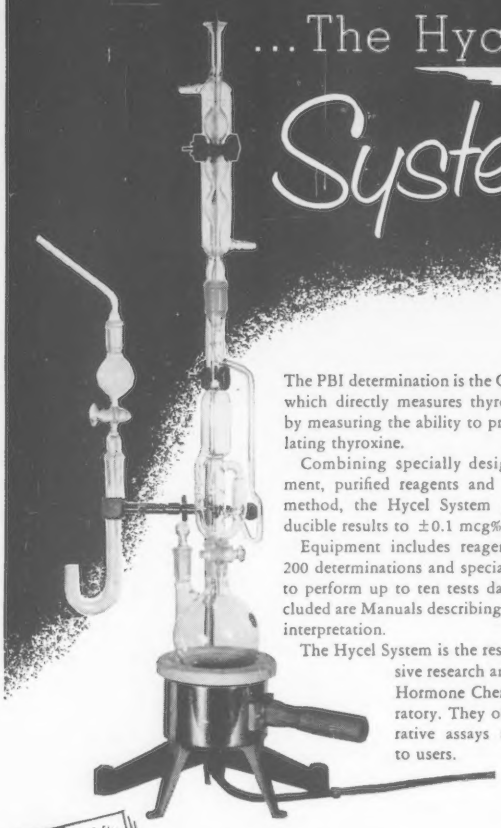

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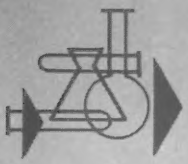

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Quantitative Electrophoresis of Serum Proteins on Paper

Moses Wurm and Frederick H. Epstein

A CONSIDERABLE NUMBER OF STUDIES devoted to the quantitative evaluation of paper-electrophoretic patterns have left doubt as to the value of the method as an accurate quantitative procedure. Some workers feel that paper electrophoresis cannot be expected to equal the reproducibility of the moving-boundary technic by virtue of its relative crudeness as compared with the sensitive nature of refractive index measurements. To others, meaningful comparisons between the two methods are impossible a priori, since the two methods measure different quantities—a mixture of protein, lipid, and mucopolysaccharide in one and protein alone in the other. Finally, the quantitative aspects of the interaction between protein and dye and the measurement of dye uptake have given rise to difficulties in interpretation (1).

The present study reports a paper-electrophoresis technic which, applied to normal human serum, affords satisfactory resolution of protein components, reduces to a minimum disturbing factors which affect quantitative reproducibility, describes a quantitative densitometric procedure which yields values comparable to those determined by electrophoresis in a moving-boundary apparatus, and establishes statistical confidence limits in the results.

It is now generally recognized that the two methods cannot be expected to yield strictly identical results. Part of this discrepancy is due

From the Research Department, Sidney Hillman Health Center, New York, N. Y.
Present address (M. W.): Department of Pathology, Saint Joseph Hospital, Burbank, Calif.

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The authors are indebted to the late Dr. Ernst P. Boas for his encouragement throughout this investigation. Thanks are also due to Prof. Kurt G. Stern, Polytechnic Institute of Brooklyn, N. Y., for the use of the moving-boundary apparatus as well as for helpful discussions and a critical reading of the manuscript.

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to the fact, already mentioned, that the two methods measure different chemical substances. Small errors are introduced on account of trailing in paper electrophoresis; diagrams obtained by moving-boundary electrophoresis, on the other hand, are also subject to difficulties in interpretation (2). In normal sera, however, the two methods should give at least reasonably similar proportions for the various serum fractions. During our early studies, we were, therefore, disturbed to find gross underestimation of the albumin fraction on densitometric evaluation of paper electrophoretic patterns. Further work led to the conclusion, reported since by a number of independent investigators (2, 3, 4), that this anomaly was caused by deviations from Beer's law at higher concentrations. Our studies suggested that Beer's law could be replaced, under these circumstances, by a logarithmic relation between protein concentration and optical density readings (5). Evidence for the existence of such a relationship will be given and discussed in the present paper.

EXPERIMENTAL MATERIAL AND METHODS

Sample

Ten consecutive pairs of determinations by the moving-boundary and paper-electrophor sis methods were carried out on the serum of a single normal donor. This was done deliberately with the purpose of eliminating the variables inherent in multiple donors. Blood samples were taken on 3 occasions during a 5-week period in order to reduce the hazards of prolonged storage. The values reported below, therefore, represent to a minimal degree the influence of biologic variation and storage effects, and largely reflect the technical variations inherent in the two procedures.

Paper-Electrophoresis Technic

The basic procedures involved in paper electrophoresis have been extensively described in the literature (6, 7, 8). The apparatus[1] used in this study (Fig. 1 [Plate 1]) was designed to keep filter-paper strips taut, horizontal, and freely suspended in a moist atmosphere. Removable strip holders made it possible to dry patterns untouched in a horizontal position. On removing holders, excess fluid in the dependent part of the strips was expressed by passage through slots. This prevented gravitational chromatographic shifts in the separated protein zones prior to drying. The large capacity—4 L.—of each buffer vessel, and the long dis-

[1] Paper electrophoresis apparatus manufactured by Standard Scientific Supply Corp., New York, N. Y.

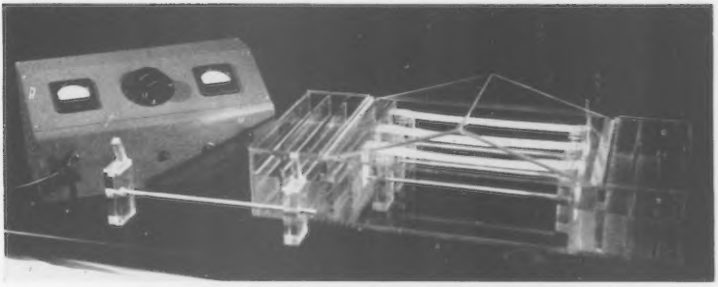

Fig. 1. Apparatus for paper-strip electrophoresis.

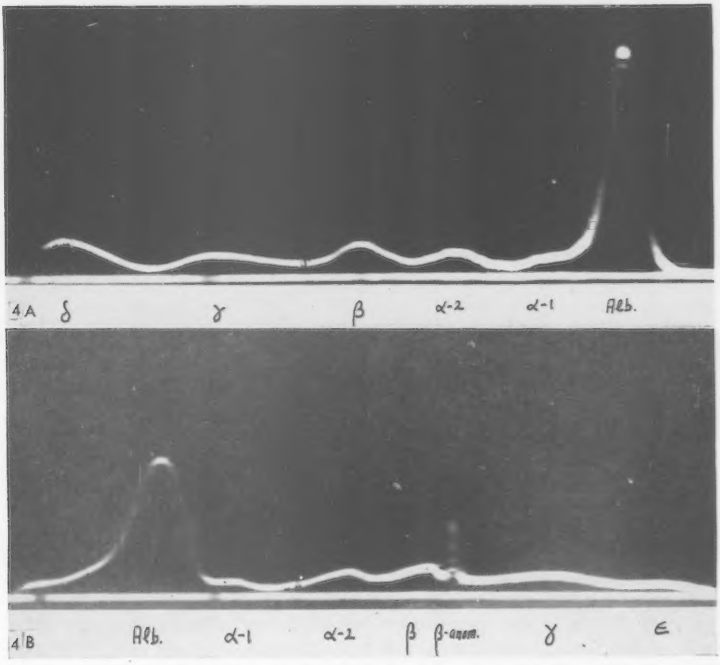

Fig. 4. *A*, moving-boundary electrophoresis diagram, ascending limb. *B*, moving-boundary electrophoresis diagram, descending limb.

Plate I

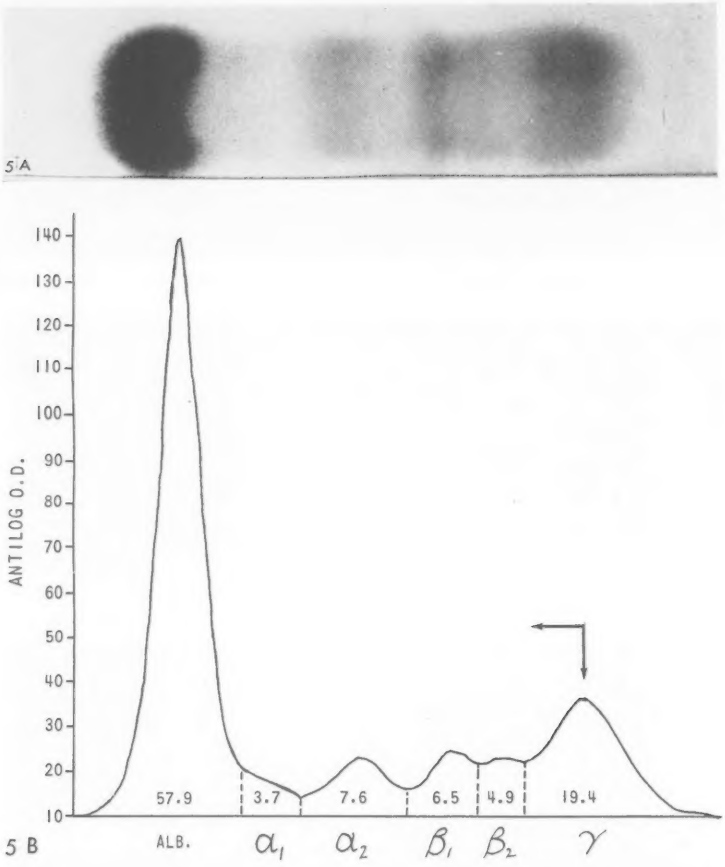

Fig. 5. *A*, protein pattern on filter paper, stained with bromphenol blue after electrophoresis for 18 hours at 160 volts, 4 ma. ***B***, corresponding protein distribution curve determined by densitometry. Values under each peak represent relative concentration in per cent of total area. *Ordinate:* antilog of optical density × 10. *Abscissa:* distance along pattern.

Plate 2

tance—40 inches—between the ends of the paper strips and each of the platinum electrodes made it possible to carry out all the determinations reported here without the need of changing the buffer as verified by repeated *p*H measurements.

All experiments were performed at room temperature using Whatman 3MM filter paper which was wetted with barbital buffer of *p*H 8.6 and ionic strength 0.05. A direct current potential of 3 v./cm from a variable voltage-regulated power supply gave a current density of 0.25 ma/cm. Although the major protein fractions could be separated in 7 hours using higher current densities, operating at the indicated current strength for 18 hours avoided excessive evaporation from the migrating surface and fluid shifts, thus producing more regular patterns.

In operation, dry strips of filter paper measuring 1.5 x 24 inches were clamped in the strip holders, the ends were dipped into the buffer solution, and the paper was allowed to become uniformly wetted by capillary movement. To eliminate the sag in the wet paper, the strip holders were adjusted without touching the paper so that all strips were equally taut and horizontal. Undiluted serum, 5 to 15 μl., was applied to the wet paper strip with a melting point capillary as a narrow band. No attempt was made to apply uniform quantities. The length of the band was approximately 0.75 inches, thus leaving a clear margin between the migrating proteins and the edge of the paper strip. The line of application was selected so that the γ-globulins remained at the origin at the completion of the run. At this time the strip holders were removed from the migration chamber without disturbing the wet paper, placed in an oven at 90°, and the paper strips allowed to dry in a horizontal position for 5 minutes. The dry strips were then removed from the supports and reheated for 5 minutes at 140° to thoroughly fix the proteins.

Staining

Protein patterns were developed by staining with Amidoschwarz 10B according to Grassmann and Hannig (9) or with bromphenol blue according to Durrum (10). The latter method was modified by increasing the dye concentration to 0.5 Gm. bromphenol blue/L. 5% acetic acid saturated with mercuric chloride. This shortened the time of staining to 5 minutes. Removal of background stain was satisfactorily accomplished by rinsing for 5 minutes each in three baths of 2% acetic acid, followed by a final rinse for 3 minutes in 2% acetic acid containing 0.5% sodium acetate. For the final drying the strip was again kept in the oven in a horizontal position at 140° until the proteins appeared as dark blue bands.

Quantitation

Relative protein concentrations were determined by transmission measurements with a photoelectric densitometer.[2] The dry, stained strip was centered over the slit diaphragm of the light source and the base line corresponding to 100 per cent transmission adjusted for a blank region on the filter paper. The strip was then advanced continuously over the scanning slit, plotting photometric values, as defined below, as the ordinate whenever a change of 0.025 units on the optical density scale was observed. A pattern, extending over a distance of 9 cm. along the abscissa, was represented by approximately eighty plotted values. A smooth curve was drawn through these points and the different fractions were demarcated by dropping perpendiculars from each of the minima to the base line. The relative amounts of the individual serum protein components were determined by planimetry of the areas enclosed under each peak.

Moving-Boundary Eectrophoresis

Experiments were carried out in a Tiselius apparatus[3] using a single-piece electrophoresis cell, barbital buffer of *p*H 8.6 and ionic strength 0.1, on serum previously dialyzed for 24 hours against this buffer. The electrophoretic diagrams recorded for the ascending limb of the cell showed the major protein components clearly resolved. The descending boundary, on the other hand, often showed less complete resolution, and the evaluation of these patterns was complicated by the presence of the so-called beta-anomaly. The values here reported are therefore based on the ascending boundaries although both boundary diagrams were recorded at the end of the run and at an intermediate stage of electrophoresis. The ascending patterns were enlarged by projection and a careful tracing made of the inner and outer edges of the curve and baseline. A mean curve and baseline were then constructed from which the relative protein concentrations were determined as described for paper electrophoresis.

OBSERVATIONS AND RESULTS

Quantitation

Direct photometry of stained paper electrophoretic patterns of human serum in transmitted light, based on the applicability of Beer's law,

[2] Model 525 Electronic Densitometer, Photovolt Corp., New York, N. Y.
[3] Portable electrophoresis apparatus manufactured by the American Instrument Co., Silver Spring, Md.

yielded values grossly different from those found by moving-boundary electrophoresis. Our results indicated a nonlinear relationship between dyestuff-protein absorbence and protein concentration. In order to determine the mathematical form of this relationship, serial dilutions of a sample of pooled human serum were prepared with normal saline, and an equal volume of each dilution was applied as a separate spot on a single strip of filter paper. These paper strips were heat-fixed at 140°, stained with either bromphenol blue or Amidoschwarz 10B, dried, and scanned in a densitometer. Figure 2 illustrates a typical series of optical density (O.D.) readings through these spots. The area integral of optical density-distance ($A \int O.D. \times d$) under each peak or total absorbence was measured by planimetry and the values plotted against the respective serum concentrations. A linear relationship was found to exist between the logarithm of protein concentration and total absorbence (Fig. 3). Similar results were obtained when the spots containing graded amounts of protein were produced by applying increasing volumes of a single serum dilution, or when samples were applied as short narrow bands. In these instances the density distributions under each peak were less symmetrical than illustrated in Fig. 2 but the total density areas were again directly proportional to the logarithm of the protein concentration (log C $\propto$ O.D.).

To construct a protein-distribution curve from the stained paper-electrophoresis pattern of serum, the above relation was expressed as C $\propto$ antilog O.D. and the antilogarithm equivalents of the optical density readings (photometric values referred to above) were plotted as a function of distance of migration. This allowed for the integration of each area directly in terms of relative concentration and eliminated the need for graphic correction with the aid of calibration curves (2, 3, 4). The use of a calibration curve presupposes that the strip used for calibration

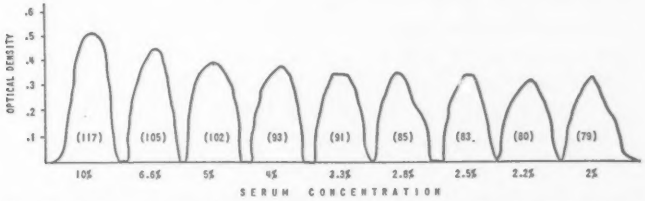

Fig. 2. Optical density readings through stained (Amidoschwarz 10B) spots produced by applying equal volumes (0.04 ml.) of serial dilutions of serum on paper. Values in parentheses represent area integrals of optical density-distance measured by planimetry.

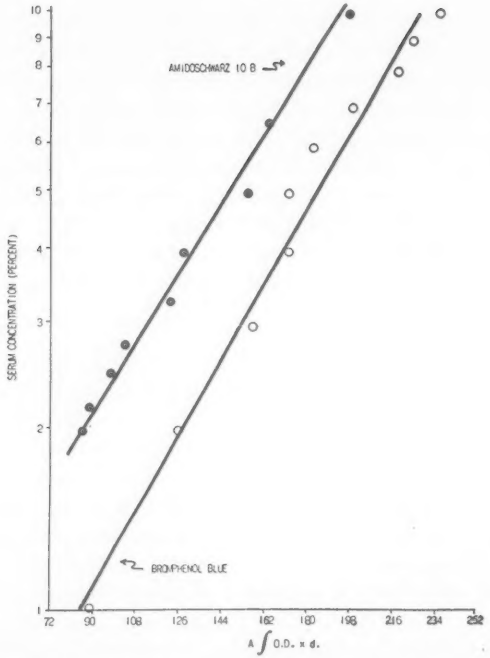

Fig. 3. Semilogarithmic plot of protein concentration and total absorbence values.

and the experimental strip are treated and stained in exactly the same way. While it may be possible to achieve this with closely standardized technics, in our experience it is difficult to reproduce identical experimental conditions from day to day or even from strip to strip. Two series of spots from a single set of serial dilutions of serum will yield straight-line semilogarithmic plots relating concentration and optical density, but the total absorbence observed in pairs of spots of equal concentration on different paper strips will be different. In our method of evaluation, slight individual variations in the paper strips, staining reactions, handling of strips or size of sample will not materially affect the result.

Contrasting values observed when a paper electrophoretic pattern was quantitated according to the above method (log C $\propto$ O.D.) and according to Beer's law (C $\propto$ O.D.) are illustrated in Table 1. With use of the logarithmic formula, relative protein concentrations are shown to correspond to the analytical results obtained with a Tiselius apparatus,

Table 1. Effect of Different Methods of Staining and Quantitation on Relative Protein Concentrations Determined by Paper Electrophoresis

Stain	*Quantitation Method*	*Relative protein concentration (%)*				
			Globulins			
		Albumin	α_1	α_2	β	γ
Bromphenol blue	Beer's law	39.8	7.0	14.3	17.4	21.6
Bromphenol blue	Log C $\propto$ O.D.	60.1	3.8	8.1	12.6	15.4
Amidoschwarz 10B	Beer's law	38.7	7.2	11.9	18.7	23.6
Amidoschwarz 10B	Log C $\propto$ O.D.	59.0	4.0	6.9	13.4	16.8
	Moving boundary	57.2	5.9	7.4	15.0	14.7

irrespective of the method of staining, and in the other case fall in the range of values frequently reported for paper electrophoresis of normal human serum (11, 12).

Comparison of Free and Paper Electrophoresis

A typical set of electrophoresis diagrams obtained in the series of 10 determinations by moving-boundary electrophoresis is reproduced in Figs. 4*A* and *B* (Plate 1). The corresponding stained protein pattern and photometric distribution curve for paper electrophoresis are shown in Figs. 5*A* and *B* (Plate 2). In half of the paper strips, a β_1- and β_2-globulin fraction could be resolved both visually and densitometrically, using both bromphenol blue and Amidoschwarz 10B. For purposes of comparison of the two methods of electrophoresis employed, the values for these fractions were combined into a single β-globulin value.

The results of all determinations and their statistical evaluation are shown in Table 2. Two sets of values are given for the moving-boundary method as calculated on the basis of the areas enclosed by the mean protein curve, and the mean and the upper baseline, respectively; use of alternative baselines significantly altered the relative values for certain protein fractions. To compensate for the small number of determinations in each series, standard errors were calculated on the basis of N-3. On statistical comparison of the two methods of electrophoresis, albumin values exhibited a high degree of identity ($t < .63$) and α_1- and β-globulin values were at variance within experimental limits ($t < 1.96$). Statistically significant differences, greater than 5% level ($t > 1.96$), were found in the γ-globulin and α_2-globulin values. While no reason for the latter is apparent, the higher values for the relative γ-globulin concentration found by paper electrophoresis were probably due to an adsorption of

Table 2. Relative Protein Concentration of a Normal Human Serum Determined by Replicate Analyses Using Paper and Moving-Boundary Electrophoresis

Determination no.[a]	*Albumin*	*Globulins*			
		α_1	α_2	β	γ
		PAPER ELECTROPHORESIS			
1 b.	63.1	4.1	7.4	11.0	14.4
2 b.	56.0	4.5	8.2	13.6	17.8
3 a.	57.9	3.7	7.6	11.4	19.4
4 b.	56.3	4.3	7.8	13.5	18.1
5 b.	60.9	2.8	7.6	13.0	15.7
6 a.	55.0	4.7	6.0	16.7	17.6
7 a.	63.5	2.5	6.2	13.6	14.3
8 b.	54.5	4.1	6.7	12.9	21.9
9 b.	55.3	4.4	7.9	12.3	20.0
10 a.	62.2	4.0	6.8	12.4	14.5
Mean ± S.E.[b]	58.5 ± 1.29	3.9 ± .27	7.2 ± .27	13.0 ± .56	17.4 ± .94
		FREE ELECTROPHORESIS (UPPER BASELINE)			
1	57.6	5.5	7.9	15.7	13.2
2	56.7	4.5	12.2	14.5	12.1
3	58.0	5.0	8.4	13.8	14.9
4	62.0	3.5	7.7	12.2	14.6
5	57.8	5.1	8.9	14.7	13.5
6	58.9	4.2	9.2	13.4	14.3
7	57.6	3.8	7.9	14.4	16.3
8	60.9	4.9	8.2	13.1	12.9
9	61.1	4.0	8.6	13.5	12.8
10	60.7	4.0	8.1	13.4	13.8
Mean ± S.E.	59.1 ± .67	4.5 ± .24	8.7 ± .47	13.9 ± .35	13.8 ± .44
		FREE ELECTROPHORESIS (MEAN BASELINE)			
1	54.6	5.0	13.0	14.8	12.5
2	54.4	6.3	9.0	16.4	13.8
3	56.8	4.2	9.1	13.3	16.6
4	54.0	5.5	9.8	14.4	16.4
5	53.5	6.2	10.5	15.5	14.2
6	54.4	5.1	10.4	14.5	15.7
7	53.5	4.9	9.3	15.0	17.2
8	56.3	5.4	9.1	14.1	15.1
9	57.7	5.3	9.5	14.0	13.4
10	55.9	4.8	9.6	14.1	15.6
Mean ± S.E.	55.1 ± .53	5.3 ± .23	9.9 ± .43	14.6 ± .31	15.1 ± .55
		FISHERS "t"			
Paper and upper baseline	.41	1.39	2.77	1.36	.346
Paper and mean baseline	2.45	4.00	5.29	2.50	2.11
Upper baseline and mean baseline	4.70	2.42	1.87	1.49	1.86

[a] a = Amidoschwarz 10B; b = Bromphenol blue.

[b] Standard errors calculated on basis of N−3 to correct for small sample size.

other protein fractions upon passing through this zone. The over-all effect, furthermore, was to yield slightly lower values for all other protein components as determined by paper electrophoresis.

DISCUSSION

Our data, as well as those of others (2, 13, 14, 15) show that excellent reproducibility can be achieved by paper electrophoresis. In order to compare this procedure with the moving-boundary method, it is equally important to consider the confidence limits expected from the latter. Our findings are in good general agreement with those reported by Bierring and Nielsen for the classical Tiselius and Antweiler apparatus (16) and those reported by Redmond (17) and Cooper *et al.* (18) for the apparatus used in this study. It appears justifiable to conclude that paper electrophoresis closely approaches but does not equal the reproducibility of the classical method.

Differences and Limitations of Methods

Reproducibility of the two methods being of a similar order does not, of course, imply that the two methods are equivalent in terms of what they measure. Paper electrophoresis has the advantage of measuring single components rather than mixtures of proteins, lipids, and mucopolysaccharides. This is counterbalanced to some extent by the disadvantage of trailing (19, 20), causing relatively greater errors in the estimation of the γ-globulin fraction. Free electrophoresis, on the other hand, is also subject to a number of limitations (2), among which the following may be mentioned: (a) that the observed values are dependent on concentration; (b) that the observed values vary with the ionic strength of the buffer used; (c) that the procedure depends upon changes in refractive index and that this nonspecific measurement is influenced by the presence of lipids and polysaccharides; (d) that buffer gradients interfere with a valid measurement of the refractive index gradient due to proteins; (e) that the resolving power of different instruments require that either the ascending, the descending, or both patterns be evaluated.

Choice of Baseline

Another uncertainty inherent in the evaluation of moving-boundary diagrams not often emphasized in the literature relates to the choice of baseline position in calculating relative protein concentrations. The Aminco apparatus is equipped with a control for varying the angle at which the schlieren diaphragm intersects the image of the light source

projected by the schlieren lens elements. Changes in this angle, in effect, control the vertical dimensions of the electrophoretic pattern and permit a more critical examination of the gradient curve. The "baseline," which is simultaneously recorded with the refractive index gradient curve, is produced by a beam of light through a narrow vertical slot in the cell-holder mask which bypasses the cell channels. As electrophoresis progresses, the initial steep boundary between serum and buffer becomes more diffuse and the boundaries of the different protein fractions appear as individual peaks in the diagram. That portion of the "baseline" which represents a superimposition of the gradient curve over the mechanical baseline appears noticeably wider on the plates, due to greater exposure. Since the mean baseline in this region approximately coincides with the upper edge of the image of the mechanical baseline, this edge is preferred as a reference line in the planimetric evaluation of the areas under the various maxima.

Criteria for Comparison

In discussing the relative merits of free and paper electrophoresis, Crook and associates (2) take the extreme view that resemblance of the data obtained by the two methods "can be regarded as nothing more than an interesting and useful coincidence." Mackay *et al.* (15), on the other hand, find that the two methods agree well in the case of normal sera and that, in various pathologic conditions, "the general protein disturbances . . . are very satisfactorily revealed by the paper technique." Part of this controversy stems from an attempt by earlier workers to assess the value of paper electrophoresis by determining how closely it could be made to correspond to data obtained by the classical method.

While there is no theoretical justification for such an attempt in a rigid sense (2), the conclusions of Mackay and co-workers (15) appear to be valid in practice and agree with our experience. There are, however, two requirements for meaningful estimations of paper-electrophoretic patterns: (a) comparable dye-binding for albumin and the different globulins, and (b) quantitative relationships between dye uptake, protein concentration, and the measurement of dye bound.

Dye-Binding Capacity

In our studies, we have assumed equal dye-binding for the different serum fractions. Cremer and Tiselius (21) originally reported that albumin bound more dye than globulin and suggested a correction factor of 1.6. Köiw and associates (22), however, showed that no constant factor

was adequate for this purpose and that each component required variable corrections. Albumin and γ-globulin have been shown to have equal affinities for binding dyes when measured directly on paper but the protein-dye complexes responded differently to elution (23, 24). Furthermore, incomplete "denaturation" can effect both a decrease and an increase in dye binding by different proteins, which in their native states respectively show a strong and weak affinity for dye anions (25, 26). Reheating the unstained patterns at higher temperature insured adequate reproducibility in our work. Durrum has also stressed the importance of adequate heating (27). Franglen and Martin (28) found marked differences in dye binding between albumin and γ-globulin though, within actual working range, the differences would seem much less marked, as shown earlier by Grassmann and Hannig (9). Hardwicke (29), using elution, found no evidence for differences in dye-binding. While it cannot be denied that gamma-globulin might have slightly less affinity for dye than albumin (15), we do not feel that serious errors result from neglecting this phenomenon in practice.

Dye-Protein Interrelations

The interrelations between protein concentration, dye binding, and dye measurement are more complex. Using elution, earlier workers found relatively excessive albumin values (7, 22, 30) explained on the basis of differences in dye binding by the different fractions, as mentioned above. Surprisingly, other workers, including ourselves, evaluating patterns by direct densitometry, frequently found albumin values considerably lower when determined by paper than by free electrophoresis. This phenomenon seemed to be due to deviations from Beer's law at higher concentrations. Thus, Grassmann and Hannig (9) found deviations from Beer's law beyond optical densities of 0.7, unless the paper was made translucent; their diagrams show that maximal absorbence in their patterns did not exceed that value. Similar findings were reported by Köiw (31). "Essential" linearity between optical density and concentration has been claimed for albumin-dye (32) and hemoglobin-dye (33) in free solutions, even though calibration curves reveal significant deviations from linearity at higher protein concentrations.

Consequently, Beer's law might appear valid for densitometric determinations of protein stains on paper, provided that only small quantities of serum, well separated, be used. In fact, Mackay *et al.* (15) obtained good results by using 10 μl. of serum, although these authors admit that the density of the albumin band in their studies frequently

exceeded the allowable upper density limit set by them. Crook *et al.* (2) find deviations from Beer's law, even though they use only 5 μl. of serum, run for 16 hours, while Griffiths (34), using as much as 100 μl., claims substantial agreement with Beer's law. Cooper and his group (18), using 5 μl. applied as spots instead of bands, scanned with a filtered, circular beam of light, also see no need to correct for deviations from Beer's law. Latner initially used Beer's law (35) but later on used a modified procedure (3). Andersch and Barbusca (4) are likewise using corrected density curves.

It seems difficult to correlate these divergent observations in a simple way, since each author uses technics differing in many details. Nevertheless, the use of the logarithmic relationship between protein concentration and optical density which we have described helps towards defining a common denominator in the densitometric evaluation of electrophoretic patterns. While Crook *et al.* (2) have proposed a hyperbolic function to describe the nonlinear relationship observed for the range of protein concentrations usually encountered in paper electrophoresis, we find that the expression $C \propto$ antilog O.D., when plotted directly, gives results which agree well with those obtained in moving-boundary electrophoresis experiments. When dealing with small quantities of protein, a linear plot of total absorbence against concentration approximates a semilogarithmic plot sufficiently to satisfy the observed data. This was confirmed in additional experiments in which quantities of serum containing 0.018–0.070 mg. protein were applied as bands and dispersed by short time electrophoresis to give average protein densities between 0.4×10^{-4} tc 1.8×10^{-4} μg./mm.^{2}. Likewise, Crook's hyperbolic and our logarithmic function give similar answers within limited concentration ranges. We do, however, believe that the logarithmic function may express a more fundamental property of stained spots on paper.

Thus, in a study of quantitative chromatography, Mori (36) has shown that

$$\text{Log C} = -\text{a} + \text{b Log L}$$

and

$$\text{Log L} = \text{c} - \text{d Log W}$$

in which C, L, and W represent, respectively, concentration, length and width of chromatographed area and where a, b, c, and d are constants. Under the experimental conditions employed, uniform distribution of material across the width of the chromatogram may be assumed and, when scanning the chromatographed zone, therefore, the percent transmission (T) may be considered equivalent to the width (W). By suitable

substitutions we find that the concentration per unit length (C/L) may be expressed as:

$$\text{Log (C/L)} = k_1 + k_2 \text{ O.D.}$$

and thus conforms with our observations. Other studies indirectly supporting the relation log C $\propto$ O.D. include the observations that log C is proportional to the area occupied by the chromatographed material (37, 38), to the maximum color density multiplied by the area (39), or to the logarithm of the length of the spot (40).

In the foregoing considerations, deviations from Beer's law were implicitly assumed to be due to a failure on the part of the stain density as recorded by the photoelectric cell, to increase in linear fashion as the concentration of protein per unit area increases. Recently, however, Franglen and Martin (28) reported experiments which indicated diminution of dye uptake with increasing protein concentration. Hardwicke (29), on the basis of similar findings, believes that this phenomenon is "probably due to the fixed coagulum becoming impermeable to the dye"; he, therefore, advises working at low protein concentrations. Evidently this situation could also account for failure to obtain linearity between protein concentration and total absorbence, irrespective of deviations from Beer's law. Even though some of Franglen and Martin's findings are difficult to explain, recalculation of their own data in terms of protein density would indicate that, under actual working conditions of paper electrophoresis, densities are usually sufficiently low to fall within a part of the curve where dye uptake is approximately proportional to protein concentration. Nevertheless, it would seem advisable to use small quantities of serum in paper electrophoresis; under our own experimental conditions, no difficulties have been experienced with 5–15 μl.

SUMMARY

1. A procedure for paper electrophoresis has been described which gives highly reproducible protein patterns with good resolution and freedom from distortions.

2. Densitometry of protein bands on paper stained with bromphenol blue or Amidoschwarz 10B reveals that the logarithm of protein concentration is proportional to optical density and that Beer's law does not apply. Electrophoretic patterns of normal human serum evaluated in this manner give values in close agreement with those obtained by moving-boundary electrophoresis.

3. Confidence limits were determined for both methods.

ADDENDUM

In a paper which appeared in this journal as our manuscript was about to be submitted for publication, Rees and Laurence maintain that Beer's law is valid within a wide range for the quantitative densitometric estimation of protein on paper (41). Our own data are not comparable since oiled paper was not used in our studies. However, Grassmann and Hannig (9), Crook *et al.* (2), and Mackay *et al.* (15) all used oiled paper and found deviations from Beer's law at optical or protein densities below those reported by Rees and Laurence as being within linear range. The data of Mackay *et al.*, in particular, are comparable with those of Rees and Laurence. The former authors find deviations from Beer's law at "protein densities" above 29 μg./cm.^{2} for Amidoschwarz 10B while the latter, according to their Figure 6, *a* and *c*, are still within linear range at "surface protein concentrations" of 100 μg./cm.^{2}. We submit that calculation of data according to Beer's law requires that the validity of this law be tested each time under the particular experimental conditions used. The logarithmic relation proposed by us, on the other hand, would seem to apply irrespective of the paper-electrophoretic or densitometric method employed.

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Blood *p*H Measurement with the Glass Electrode

Study of Venous and Fingertip Blood

*Samuel Natelson and Norbert Tietz**

OBSERVATIONS MADE DURING the routine estimation of blood *p*H with the glass electrode noted that specimens of fingertip blood often tended to drift rapidly toward the alkaline side when exposed to air at 37° or showed a slower drift in this direction under oil. This change was noted to be slower with specimens of venous blood.

The *p*H of blood taken from fingertip or vein under oil and cooled rapidly to room temperature, did not tend to drift. When exposed to air at room temperature, the *p*H of fingertip blood drifted more rapidly to the alkaline side, than did venous blood.

In patients with acidemia, with low CO_2 plasma levels, this drift was not marked or did not occur at room temperature or at 37°, either under oil or exposed to air.

It was suspected that, since with fingertip blood the tendency to rupture erythrocytes is greater than when blood is drawn from the vein and the proportion of tissue juice is greater in fingertip blood than in venous blood, the difference between venous and fingertip blood lay in their respective carbonic anhydrase content.

A study was therefore set up to test the effect of carbonic anhydrase and its inhibitor, Diamox (1) (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide), on the drift of *p*H in the blood and plasma from normal individuals.

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* Present address: Reid Memorial Hospital, Richmond, Indiana.

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METHOD AND MATERIALS

Carbonic Anhydrase (1 mg./ml.)

Twenty-five mg. of crystalline carbonic anhydrase[1] was dissolved in 25 ml. of normal saline. This solution contained 99 units/ml. when assayed by the method of Philpot (2).

Diamox (40 mg./100 ml.)

Forty mg. crystalline Diamox[2] was added to 100 ml. of normal saline and shaken on a Kahn shaker until dissolved. This solution would completely inhibit the carbonic anhydrase solution, volume for volume within experimental error, when tested by the method of Philpot.

Apparatus

The *p*H for venous serum was measured in the instrument shown in Fig. 1, using the Beckman electrodes. The glass electrode used was approximately 5 mm. in diameter with a long shielded lead, as used in gastric *p*H measurements. The micro calomel electrode was the fiber type. The micro sleeve-type calomel electrode was also occasionally used, with no observable difference in results. The *p*H meter used was, at first, the Model G Beckman *p*H meter. Although relatively gross differences were measurable with this instrument (± 0.04 *p*H units), for more accurate measurements (± 0.01 *p*H units) an instrument previously described was resorted to (3).

PROCEDURE

Venous Blood

Sterilized mineral oil was added to a sterile syringe, the syringe was lined with oil, and most of the oil was expelled. The syringe was then kept wrapped in cloth in a refrigerator until used. A needle was fitted to the syringe, some oil was expelled, and the blood was drawn from the vein without applying suction but drawing the barrel back as fast as the blood would flow. The needle was then removed and the blood added under the mineral oil contained in a test tube which had been previously kept in the refrigerator. The blood was allowed to clot and then centrifuged for 15 minutes. The serum and oil was poured over to a second cooled tube.

Five milliliters of serum were removed with a syringe and transferred to the container shown in Fig. 1. When *p*H measurements were taken at

[1] Worthington Biochemical Company, Freehold, N. J.
[2] Lederle Company, Pearl River, N. Y.

37°, the container of Fig. 1 was kept in a 37° bath, and after the serum was added a thermometer was inserted in place of the calomel electrode. The container was rocked by attachment to an acentric wheel on a motor at 80 rpm for 2–3 minutes to allow for temperature adjustment. The thermometer was then replaced by the calomel electrode and *p*H measurements taken. The initial values shown on the graphs are *p*H measurements taken at room temperature and corrected for the temperature

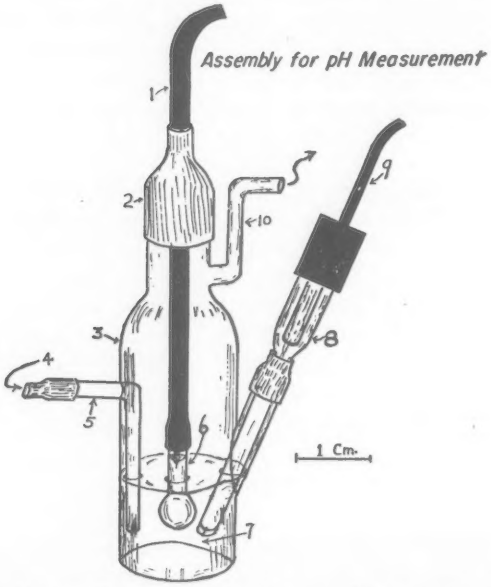

Fig. 1. Assembly for *p*H measurement under controlled atmosphere and at constant temperature. *1*, shielded lead from *p*H meter; *2*, rubber tubing; *3*, glass container; *4*, inlet for air or 4% CO_2; *5*, glass bubbling tube; *6*, glass electrode; *7*, sample being measured; *8*, calomel electrode; *9*, lead to *p*H meter; *10*, outlet tube.

coefficient of blood (4). The first measurement at 37° is timed from the moment the blood reached that temperature. The remaining measurements were timed from that period.

Measurements under oil were made with a layer of approximately 1 cm. of oil above the serum. Measurements under 4 per cent CO_2 were taken by bubbling through the serum (no oil) residual expired air of the subject's lungs, collected in a large plastic bladder which was rinsed repeatedly with residual expired air before filling. The air was quickly

flushed out through the bubbler with the residual expired air, and then the bubbles were passed through at approximately two per second during the measurement. Measurements under air were taken by bubbling air through the bubbler at 1 bubble per second.

Fingertip Blood

For fingertip blood, the blood was collected as described previously (4) with a tube of 1.5 mm. bore. For measurements in air it was transferred promptly to the one-drop electrode (4). Measurements under oil were made by adding oil to the cup of the one-drop electrode and adding the freshly drawn blood under the oil. For measurements at 37° the *p*H meter with electrodes was kept in a 37° incubator for 1 hour before and during the experiment. Blood was drawn from the fingertip and transferred directly to the one-drop electrode.

Addition of Carbonic Anhydrase and Inhibitor

In the experiments described, 0.1 ml. of carbonic anhydrase solution (100 μg.) was added to 5 ml. of serum unless otherwise specified. For fingertip blood the same ratio (50:1) was used, or 2 μl. for 0.1 ml. of whole fingertip blood. When Diamox was used, 0.2 ml. was added to 5 ml. serum, or a ratio of 25:1. For 0.1 ml. of fingertip blood, 4 μl. of Diamox solution were used. When carbonic anhydrase and Diamox were used together, the Diamox was always added first. When Diamox alone was added to fingertip blood, a calibrated collecting tube was used. The Diamox was added first and blood was drawn to the 0.1-ml. mark and the two mixed, allowing the blood to run back and forth in the tube.

Crushed erythrocytes were the saline-washed erythrocytes of the patient whose serum was being tested. The cells were hemolyzed by mechanical homogenization in a test tube. To 5 ml. of venous serum, 0.05 ml. of crushed red cells was added, or in a ratio of 100:1. This same ratio was used for fingertip serum when crushed red cells were added. The crushed-red-cell preparations contained approximately 1000 carbonic anhydrase units per milliliter or about ten times that of the carbonic anhydrase solution. When Diamox solution was used to inhibit the erythrocyte carbonic anhydrase activity, 0.5 ml. was used for 5 ml. of serum and 0.01 ml. for 0.1 ml. of fingertip serum.

Readings

At intervals *p*H readings were taken as shown in Figs. 2, 3, and 4. Only initial and terminal values are listed, however, in Tables 1, 2, and

Table 1. MEAN *p*H DRIFT OF SERUM AND BLOOD FROM 8 HEALTHY MALES IN AIR (AFTER 50 MINUTES)

Sample	*Temp.* (°C.)	*pH Initial*	*Initial CO_2 content* (mM/l.)	*Final pH*	*Final CO_2 content* (mM/l.)
Fingertip serum	25°	7.38 ±0.03	19.5 ±1.5	7.82 ±0.03	17.4 ±2.0
Fingertip serum	37°	7.38 ±0.03	19.5 ±1.5	7.96 ±0.03	16.1 ±2.1
Venous serum	37	7.42 ±0.01	24.1 ±1.8	7.77 ±0.02	20.2 ±1.9
Fingertip serum + Diamox	37	7.42 ±0.02	19.3 ±1.3	7.76 ±0.02	16.9 ±1.1
Venous serum + crushed red cells	37	7.38 ±0.03	22.2 ±1.4	7.93 ±0.03	17.8 ±2.3
Venous serum + crushed red cells + Diamox	37	7.40 ±0.02	22.2 ±1.4	7.78 ±0.02	19.9 ±1.5
Venous serum + carbonic anhydrase	37	7.40 ±0.02	24.1 ±1.8	7.94 ±0.04	18.9 ±2.0
Venous serum	25	7.42 ±0.02	24.1 ±1.8	7.72 ±0.02	21.0 ±1.9

Table 2. MEAN *p*H DRIFT OF VENOUS SERUM FROM 5 HEALTHY MALES UNDER 4% CO_2 (AFTER 50 MINUTES) AT 37°

Addend to venous serum	*Initial pH*	*Initial CO_2 content* (mM/l.)	*Final pH*	*Final CO_2 content* (mM/l.)
None	7.40 ± 0.02	24.8 ± 1.1	7.57 ± 0.02	25.2 ± 1.3
Diamox	7.41 ± 0.02	24.7 ± 0.8	7.54 ± 0.02	24.7 ± 1.5
Carbonic anhydrase	7.40 ± 0.02	24.7 ± 1.1	7.54 ± 0.02	24.8 ± 0.9
Carbonic anhydrase + Diamox	7.40 ± 0.03	24.8 ± 1.2	7.56 ± 0.03	25.0 ± 1.4
Crushed red cells	7.38 ± 0.03	24.0 ± 1.5	7.54 ± 0.02	24.8 ± 1.8
Crushed red cells + Diamox	7.39 ± 0.03	24.1 ± 1.3	7.55 ± 0.03	23.9 ± 1.6

4% CO_2 residual expired air of the subject.

Table 3. MEAN *p*H DRIFT OF VENOUS SERUM FROM 5 HEALTHY MALES UNDER MINERAL OIL (AFTER 50 MINUTES)

Addend to venous serum	*Temp.* (°C.)	*Initial pH*	*Initial CO_2 content* (mM/l.)	*Final pH*	*Final CO_2 content* (mM/l.)
None	20	7.40 ± 0.02	24.6 ± 1.3	7.40 ± 0.01	24.4 ± 1.5
None	25	7.40 ± 0.02	24.6 ± 1.3	7.43 ± 0.02	24.4 ± 1.4
None	37	7.40 ± 0.02	24.6 ± 1.3	7.56 ± 0.03	24.3 ± 2.4
Carbonic anhydrase	37	7.40 ± 0.02	24.6 ± 1.4	7.55 ± 0.03	24.1 ± 2.1
Diamox	37	7.42 ± 0.03	24.4 ± 0.9	7.53 ± 0.03	24.3 ± 2.0
Diamox + Carbonic anhydrase	37	7.41 ± 0.03	24.4 ± 1.7	7.53 ± 0.02	24.0 ± 2.2
Crushed red cells	37	7.37 ± 0.03	24.0 ± 2.1	7.52 ± 0.02	23.6 ± 2.5

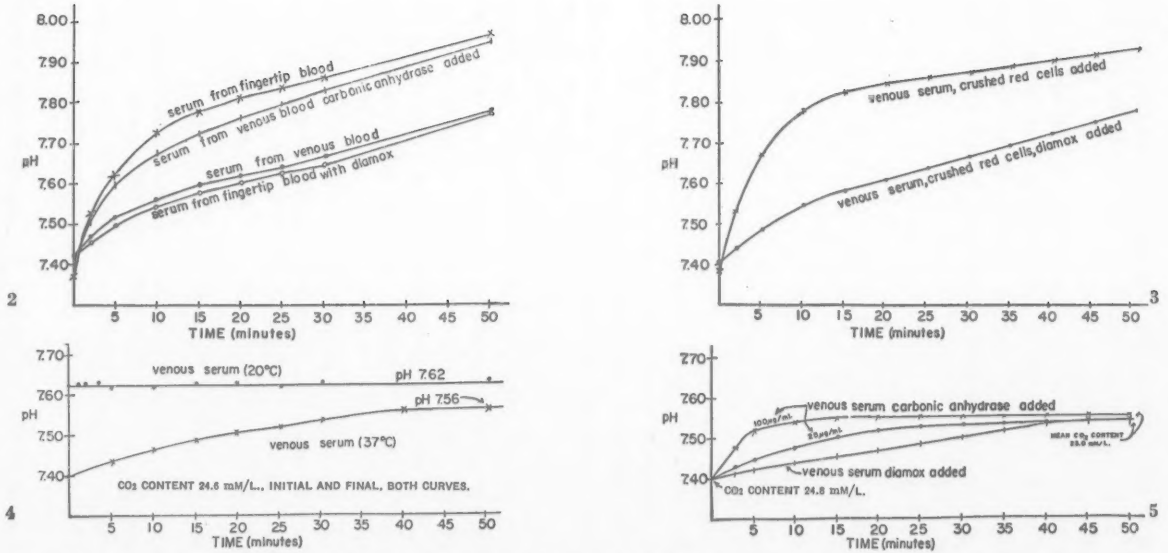

Fig. 2. Drift of blood *p*H under air (37°). Comparison of fingertip blood with venous blood from the same patient. Note that fingertip blood drift resembles that of venous blood when Diamox is added and venous blood drift resembles that of fingertip blood when carbonic anhydrase is added. Mean values for blood from 5 healthy individuals. **Fig. 3.** Effect of adding crushed red cells on the drift of venous serum *p*H in air (37°). Note that crushed red cells simulate the effect of carbonic anhydrase added (Fig. 2), and the inhibiting effect of Diamox. Mean values for serum from 8 healthy individuals. **Fig. 4.** Effect of temperature on rate of *p*H drift of serum under mineral oil. Values are uncorrected for temperature coefficient of serum. When *p*H at 20° is corrected to 37°, 7.62 is equivalent to 7.40 for the instrument used in our laboratory. **Fig. 5.** Effect of carbonic anhydrase on *p*H drift of venous serum under residual expired air (37°) of the individual's serum being tested. Diamox added to counteract any possible effect due to hemolysis. Mean values for 5 healthy individuals.

3. The general shape of these curve simulated those in Figs. 2, 3, 4, and 5. The CO_2 content values used were obtained at the beginning and end of 50 minutes, as shown in the tables.

The CO_2 content was estimated on a micromanometric gasometer previously described (5, 6). For capillary blood this determination was made on plasma after centrifuging, before and after the *p*H determinations. The venous and capillary blood was drawn from apparently healthy blood donors.

DISCUSSION

It is apparent from Fig. 2 that when a carbonic anhydrase inhibitor is added to fingertip blood a marked reduction in the rate of drift in air is observed. When carbonic anhydrase is added to venous serum the rate of drift may be made to simulate that of fingertip blood (Fig. 2). This drift in air is due in part to loss of CO_2, as seen from Table 1. Figure 3 shows further that crushed red cells may be substituted for carbonic anhydrase with similar results. It is to be noted that at room temperature the *p*H of both fingertip blood and venous blood or serum will drift rapidly toward the alkaline side when exposed to air.

Under oil, which was found not to inhibit the enzyme activity, the *p*H of venous serum will drift at 37° to a *p*H of 7.56, as shown in Fig. 4, while at 20° no such drift occurs. This suggests that a practical method of performing this test is to allow the blood, collected under oil, to cool rapidly to room temperature, and to perform the test under oil on the separated serum. This necessitates a temperature correction determined for the particular instrument used. We prefer to compare the *p*H of the unknown serum to that of a healthy control, as discussed in an earlier publication (4).

Since no change in CO_2 content occurs when measurements such as those shown in Fig. 4 are made, it is suggested that the over-all change taking place in the serum may be represented as follows, equilibrium being approached from the left to right:

$$H\overline{CO}_3 \rightleftharpoons \overline{OH} + CO_2 \text{ (dissolved)}$$

Thus, some HCO_3 ion is replaced by $\overline{OH}$ ion, the serum becoming more alkaline. The dissolved CO_2 has no effect on the *p*H. The reason for lack of drift observed in severe acidemia with low CO_2 content then becomes obvious since only small amounts of bicarbonate are available for production of hydroxyl ions.

This same phenomenon is observed when the serum is equilibrated

with residual expired air, referred to in Fig. 5 as 4 per cent CO_2. It is to be noted that the CO_2 content remains constant while the *p*H drifts to a mean value of approximately 7.55. Carbonic anhydrase accelerates this drift but does not change the point of equilibrium as would be normally expected. Tables 2 and 3 indicate that the effect of carbonic anhydrase is to accelerate the attainment of equilibrium while Diamox acts only to inhibit the action of carbonic anhydrase.

These experiments may be interpreted as indicating that in the body the *p*H of 7.40 normally found is not a point of equilibrium but a dynamic state maintained by a balance between the rate of bicarbonate formation, the rate of the splitting of the bicarbonate, and the rate of excretion of CO_2. They may also be interpreted as indicating that certain changes take place in serum at 37° when removed from the living organism and placed on a glass-electrode system. The authors are inclined to accept the former explanation since the human organism as a whole is in a dynamic state and not at equilibrium.

SUMMARY

A study has been described to explain the tendency of fingertip blood from normal individuals to drift to alkaline *p*H more rapidly than the blood of venous serum at 37°. This occurs even under oil and without loss of CO_2. Evidence is offered to indicate that this phenomenon is due to the release of carbonic anhydrase in greater concentrations into the serum when the finger is punctured than when venipuncture is performed. An explanation is offered for the fact that serum from patients in acidemia with low CO_2 content will not drift at 37°.

It is suggested that blood *p*H determinations be made by collecting the specimen under oil, cooling rapidly to room temperature, and performing the determination under oil on the separated serum.

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Vitamin B_{12} Excretion as Index of Hepatic Disorder

Herman Baker, I. Pasher, H. P. Dolger, and H. Sobotka

DURING A STUDY concerning the role of vitamin B_{12} in diabetes (1), it was noted that a number of cases in this study failed to excrete more than 10 μg. of a 50-μg. intramuscular load dose of the vitamin. All of these patients had a history of liver disease or some form of hepatic insufficiency.

The storage of vitamin B_{12} by the liver has been studied extensively by Meyer *et al.* (2) and by Glass *et al.* (3) using radioactive Co^{60} as tracer for the vitamin. They demonstrated that the radioactive material accumulates in the liver and can be measured with a directional scintillation counter over this organ. This was especially prominent in patients with pernicious anemia when a potent source of intrinsic factor was given at the same time.

Rachmilewitz *et al.* (4) studied cases of acute hepatitis and cirrhosis of the liver and found that in these cases, the vitamin B_{12} content of the serum was very high; with subsidence of jaundice, the B_{12} concentration decreased to normal levels. This study points to the additional binding of B_{12} in liver disease and indicates the importance of a healthy liver for B_{12} storage. In liver disease the liver B_{12} stores are apparently depleted; hence the increased bound B_{12} in the serum.

This paper reports the decreased excretion of intramuscular B_{12} in cases of liver involvement and the gradual increase to normal levels during the period of convalescence.

MATERIAL AND METHODS

The assay of vitamin B_{12} was carried out using three different organisms; *Lactobacillus leichmannii*, *Euglena gracilis*, and *Ochromonas malhamensis*. These organisms were used in order to compare the B_{12} excretion values, using independent systems. The materials and methods have been outlined by Baker *et al.* (5).

From the Department of Chemistry, Mount Sinai Hospital, New York, N. Y.
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RESULTS AND COMMENTS

Table 1 shows the clinical diagnosis and comparative assay results of this study. The B_{12} values with *L. leichmannii* are higher than with the other two organisms. This is due to the increased utilization of desoxyribosides present in the urine, a point which has been treated by Baker *et al.* (5). These results point to the increased absorption of vitamin B_{12}, after administration of a 50-μg. intramuscular dose, by patients with clinical diagnosis of liver involvement.

In some cases, the results do not agree with the clinical diagnosis of convalescence, and it is this point which makes the excretion of B_{12} a valuable aid in the clinical evaluation of convalescence in liver disease. It can be noted that some patients who were considered clinically convalescent, still did not excrete normal amounts of the vitamin.

Table 1. VITAMIN B_{12} EXCRETION EIGHT HOURS AFTER A 50 μg. INTRAMUSCULAR LOAD DOSE

Patient	*Age*	*Sex*	*Diagnosis*	*B_{12} excreted (μg.)*		
				Ochromonas malhamensis	Euglena gracilis	Lactobacillus leichmannii
H.Z.	30	M	Normal control	39.8	36.2	50.2
H.D.	45	M	Normal control	33.5	26.0	55.6
R.S.	33	M	Normal control	23.8	24.5	48.7
A.W.	46	M	Obstructive jaundice, convalescent	0.6	0.5	1.2
G.G.	28	M	Pneumonia and hepatitis	1.0	2.4	3.0
L.P.	65	F	Jaundice	1.9	0.7	3.8
H.J.	48	M	Lobar pneumonia, recovered	2.5	4.2	8.3
R.V.	48	M	Pneumonia	3.6	5.6	9.9
A.V.	26	M	Portal cirrhosis	4.1	6.9	8.5
H.P.	37	M	Cirrhosis	4.9	9.2	23.4
S.S.	21	F	Bronchopneumonia	5.6	2.4	6.8
L.L.	45	F	Liver disease	6.3	11.6	16.8
A.D.	58	F	Biliary tract disease	8.1	8.1	14.4
P.R.	68	F	Bronchopneumonia	8.6	4.8	13.2
A.R.	32	M	Schistosomiasis	9.3	9.5	12.5
A.K.	21	M	Infectious hepatitis, convalescent	10.0	16.1	19.5
C.C.	54	M	Ketosis	1.0	2.9	10.6
V.W.	53	F	Pancreatitis	1.5	1.7	5.0
N.R.	35	M	Diabetic retinopathy	3.6	4.8	6.6
T.K.	46	M	Angina pectoris	4.1	7.8	10.8
K.H.	70	M	Pulmonary emphysema	5.2	3.6	8.9
W.W.	18	M	Heart disease	8.1	10.2	20.4
A.A.	30	F	Neurologic disease	9.2	5.5	11.5

Vitamin B_{12}, being a normal metabolite, would not make for undue metabolic derangement, such as can be caused by the various methods used for chemical evaluation of liver function, and can be the indicator system of choice in judging whether a patient is considered clinically cured. A high excretion of B_{12} after a load test indicates that the liver has again become capable of storing B_{12} and that the increased amounts of B_{12} from injection are no longer required for normal metabolic activity of the organ.

Four cases included in this study were convalescing from attacks of pneumonia. The role of the liver in pneumonia infections is well known, and as this study shows, these patients have a decreased excretion of B_{12}, possibly due to the effect of the infection upon the liver. None of the patients with liver disease, except some convalescing, excreted more than 10 μg. of the 50 μg. load dose, as measured by the *Euglena* and *Ochromonas* assay methods. From 23 cases reported, 20 cases excreted below 10 μg. of vitamin B_{12}. Thirteen of these had history of liver involvement. Seven cases without liver involvement excreted below 10 μg.; the 3 normal controls all excreted between 24–40 μg. of the load dose.

This study is being extended and the various types of liver involvement are being categorized to determine the extent to which B_{12} excretion is correlated with liver involvement. The technic offers an aid in detecting how extensive is the liver damage and to what point convalescence has progressed.

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Plasma Fibrinogen Determination

A Rapid Titration Method

Arthur A. Rosenberg

THE DETERMINATION OF PLASMA FIBRINOGEN is indicated when excessive and continuous bleeding occurs, especially in obstetrics, following extensive surgical procedures, and in advanced liver disease. The rapid method outlined below, a modification of the procedure described by Schneider (1), is practical for determining the presence and extent of afibrinogenemia.

Reagents

Physiologic saline.

0.25% Aqueous methylene blue.

Thrombin, topical,[1] *100 units per ml.:* Remove cap and rubber stopper from a vial containing 1000 units powdered thrombin. Add 10 ml. distilled water to the vial and rotate gently for a few minutes to dissolve the powder. Insoluble material will not interfere with the test. The reconstituted material is good for 48 hours when stored in refrigerator and removed only for use.

Specimen

Centrifuge an oxalated blood specimen for 5 minutes and separate plasma (at least 1.5 ml. plasma is required). Either potassium oxalate or ammonium-potassium oxalate may be used.

METHOD

1. Pipet 1.0 ml. saline into each of ten 12 × 75 mm. test tubes held in a rack.

From the Laboratory Service of the Veterans Administration Hospital, Albany, N. Y.
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[1] Thrombin, topical, is available from Parke, Davis & Co. or The Upjohn Co.

2. Add 1.0 ml. plasma to the first tube and mix well.
3. Serially dilute 1.0 ml. from the first tube through the remaining nine tubes. Plasma dilution equivalents are 2, 4, 8, 16, etc.
4. Add 1 drop of 0.25% methylene blue to each tube.
5. Add 0.2 ml. thrombin solution to the first tube. Remove the tube from the rack at once, and shake gently for about 2 seconds; return the tube to the rack and proceed with each tube in the same manner.
6. Permit rack of tubes to stand undisturbed at room temperature for 5 minutes.
7. Pipet 2 ml. saline into each tube. Do not shake rack or tubes.
8. Read at once the highest dilution showing a definite clot. Most of the methylene blue will be retained by the clot, yielding a tube with a two-hue color phase. The methylene blue tends to distribute itself evenly through the fluid in the tube when no clot is present and a one-hue color phase results.
9. Report the highest dilution with a clot as a titer: i.e., Fibrinogen titer = 128.

A normal plasma control should be set up at the same time.

INTERPRETATION

The plasma fibrinogen titer reflects the available coagulable protein semiquantitatively. Parallel quantitative determinations of fibrinogen protein nitrogen (2) have shown that when a titer of 128 or higher is obtained fibrinogen content is in excess of 200 mg./100 ml. A titer of 64 indicates fibrinogen present in a concentration of 100–200 mg./100 ml. A titer of 32 or less indicates fibrinogen under 100 mg./100 ml. Abnormal bleeding may occur when the fibrinogen level approaches a titer of 32 or less.

DISCUSSION

Frequent determinations of fibrinogen titer may be useful in following the therapeutic administration of whole blood or blood components in hemorrhagic situations where low or absent plasma fibrinogen is a temporary or continuing complication. In emergency situations the titer quickly reflects available fibrinogen, and eliminates need for more laborious and time-consuming biochemical method.

Methylene blue is added only to permit unstrained visualization of the clot endpoint.

Time and character of clotting, test factors employed by Bonsnes (3), are not utilized in the method herein described.

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SECOND INTERNATIONAL CONGRESS ON CLINICAL CHEMISTRY

The Second International European Congress on Clinical Chemistry will take place in Stockholm, Sweden, August 19–23, 1957. Free papers will be read but some actual topics in the field will be reviewed by invited specialists. The Congress will be combined with an international exhibition of laboratory instruments. Organizing committee: Bertil Josephson, chairman, St. Erik's Hospital, Stockholm; Kjell Agner, general secretary, Serafimer Hospital, Stockholm; Bo Norberg, treasurer, Sabbatsberg's Hospital, Stockholm; Ingemar Jungner, exhibition manager, Epidemiologic Hospital, Stockholm. The address of the Congress is Box 12024, Stockholm 12.

Further information about the Congress will be published and circularized later.

Serum Protein Analysis by Electrophoresis and by the Wolfson-Cohn Chemical Method

Comparative Study

Nils Eriksen, Lester D. Ellerbrook, and Stuart W. Lippincott

CONSIDERABLE TIME AND EFFORT has been devoted to the problem of fractionating serum protein simply, reliably, and rapidly, and at the same time in conformity with the free electrophoretic separation into albumin and α-, β-, and γ-globulins. Much progress has been made, but discrepancies remain, especially in connection with α- and β-globulins. A few years ago Wolfson, Cohn, *et al.* (1) devised a relatively simple salting-out procedure for estimating the several serum-protein fractions, in a 1-ml. sample. The method involved four direct determinations: total protein, albumin, albumin plus α-globulin, and γ-globulin. All protein concentrations were determined by a modification of the Weichselbaum biuret method. Total globulin, α-globulin, and β-globulin were determined by calculation of differences between appropriate quantities. Popper *et al.* (2), using essentially this method, investigated serum-protein partition in a series of cases dominated by liver diseases. They found that the chemical (Wolfson-Cohn) albumin, total-globulin, and γ-globulin values, but not the α- and β-globulin values, were well correlated with the corresponding electrophoretic values.

This paper presents our appraisal of a slightly modified Wolfson-Cohn procedure, and an attempt at clarification of the observed discrepancies

From the Department of Pathology, School of Medicine, University of Washington, Seattle, Wash.

Present address (S. W. L.): Brookhaven National Laboratory, Upton, N. Y.

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between the chemical and electrophoretic results. The work was divided into three parts: (a) a statistical comparison of results obtained by simultaneous analyses of serum by the two methods; (b) electrophoretic characterization of chemical fractions derived from selected serum specimens; and (c) a study of the deficiency of the usual method of calculating absolute protein concentrations from electrophoretic patterns, as revealed by determinations of total serum solids retained by a dialysis membrane.

I. COMPARISON OF CALCULATED CONCENTRATIONS

The comparison between the electrophoretic and chemical methods was based upon serum specimens obtained for the purpose of evaluating proposed diagnostic tests for cancer. Of these sera, which were routinely analyzed for total protein, albumin, and the three globulin components by the chemical method, 100 were somewhat arbitrarily selected for electrophoretic analysis. The blood was drawn from fasting adults, of whom 30 had malignant neoplasms, 8 had benign neoplasms, 34 had miscellaneous nonneoplastic diseases, and 28 had no known diseases (healthy controls). The specimens provided dispersions of protein concentrations desirable for a comparison of methods, but the results may be slightly biased because the specimens did not represent a random sample of pathologic conditions.

Methods

Chemical Fractionation

We used the 1948 Wolfson-Cohn method (1) as amended for the determination of albumin (3), with the following additional modifications:

1. We used a biuret reagent containing three times as much $CuSO_4$ and nine times as much NaOH as that used by Wolfson, Cohn, *et al.* This revised reagent is insensitive to the presence of small amounts of $(NH_4)_2SO_4$, an unavoidable contaminant in the γ-globulin precipitate.

2. We used standard solutions and reagent blanks corresponding in salt composition to the unknown solutions, whereas Wolfson, Cohn, *et al.* used a common standard solution for all determinations and a reagent blank containing the precipitating salt only in the determination of albumin. In our experience the color of the reagent blank was affected not only by Na_2SO_3, used to precipitate the total-globulin fraction, but also by Na_2SO_4, used to precipitate β- plus γ-globulins; and, in addition, the intensity of the color reaction between the standard protein (human albumin) solution and biuret reagent (theirs or ours) was increased by the presence of Na_2SO_3 or Na_2SO_4.

The blanks and standards were prepared fresh daily and the tests were read in 19-mm. cuvets at 550 mμ in a Coleman Junior Spectrophotometer against the appropriate reagent blanks.

Electrophoresis

Analyses were carried out in the commonly used *p*H-8.6 barbital buffer in the Pearson model of the standard Tiselius apparatus, at a bath temperature of 0°, a field strength of 8.9 v./cm., and a serum dilution of approximately 1:5. After 130-minute runs, patterns were recorded by the Longsworth scanning method, projected with 3-times enlargement, and traced. The tracings were subdivided by dropping a perpendicular to the baseline from the low point between adjacent peaks. Areas below the peaks were measured planimetrically. Absolute concentrations were calculated by proportioning the total-protein value (Wolfson-Cohn biuret method) over the whole pattern area, exclusive of the boundary anomaly. Both ascending and descending patterns were measured but, since there proved to be practically no difference between the two insofar as correlation with chemical values is concerned, only descending electrophoretic values are considered hereinafter. For comparison with the chemical α-globulin value, electrophoretic α_1- and α_2-globulin values for each serum were combined. No attempt was made to subdivide the α_2-globulin complex.

Statistical Analysis

Correlation coefficients were calculated according to the product-moment method, and standard errors of estimate of chemical values predicted by the regression-line equations were calculated from the formula $S_y = s_y\sqrt{1 - r^2}$, where s_y is the standard deviation of a set of chemical values and r is their correlation coefficient with respect to the corresponding electrophoretic values.

The mean difference (algebraic signs ignored) between corresponding electrophoretic and chemical values was calculated for an index of numerical agreement, which is not revealed by the correlation coefficient or standard error of estimate.

Results

Albumin

The correlation was good, r being 0.926 and S_y being 0.245 (7.4 per cent at the electrophoretic mean) over an electrophoretic-value range of 1.75–4.39 Gm./100 ml. The chemical values were usually slightly higher than the electrophoretic values, and the mean difference was 0.21 Gm./100 ml.

α-Globulin

The correlation was poor, r being 0.525 and S_y being 0.208 (17 per cent at the mean) over the range 0.78–2.24 Gm./100 ml. Excepting three cases, the electrophoretic values were higher than the chemical values, and the mean difference was 0.51 Gm./100 ml.

β-Globulin

The correlation was poor, r being 0.556 and S_y being 0.226 (22 per cent at the mean) over the range 0.71–1.81 Gm./100 ml. Excepting nine cases, the chemical values were higher than the electrophoretic values, and the mean difference was 0.33 Gm./100 ml.

γ-Globulin

The correlation was good, r being 0.914 and S_y being 0.158 (17 per cent at the mean) over the range 0.18–2.39 Gm./100 ml. The chemical values were usually slightly higher than the electrophoretic values, and the mean difference was 0.14 Gm./100 ml.

Comment

Correlation coefficients obtained by Popper *et al.* (2) were 0.874, 0.166, 0.207, and 0.889 for albumin and α-, β-, and γ-globulin, respectively. Our coefficients for albumin, α-globulin, and β-globulin were significantly higher than theirs ($p < 0.02$, 0.001, 0.001, respectively). The differences between types of cases studied probably account in part for the observed differences between corresponding correlation coefficients.

The poor agreement between electrophoretic and chemical α-globulin values, considered together with certain results presented in Part II of this paper, led to a statistical comparison of the chemical α-globulin values with the electrophoretic α_2-globulin values, and of the chemical albumin values with the electrophoretic albumin-plus-α_1-globulin values. The chemical α-globulin values proved to be as well correlated with the electrophoretic α_2-globulin values ($r = 0.527$) as with the electrophoretic total-α-globulin values, and the numerical agreement was much better (mean difference = 0.17 Gm./100 ml.) than in the previous comparison. The chemical albumin values, as might be expected from a consideration of the relative magnitudes involved, were practically as well correlated with the electrophoretic albumin-plus-α_1-globulin values ($r = 0.921$) as with the electrophoretic albumin values, but the numerical agreement was not so good (mean difference = 0.40) as in the previous comparison, all except four of the electrophoretic values having been higher than the chemical values.

II. ELECTROPHORETIC CHARACTERIZATION OF CHEMICAL FRACTIONS

Aware of potential difficulties of interpretation arising from the initial conditions of separation and the subsequent manipulation required to prepare the fractions for electrophoresis, we characterized electrophoretically one or more of the Wolfson-Cohn fractions derived from several serum specimens, none of which were included in the series of Part I. We studied particularly the albumin and γ-globulin fractions, and did not attempt to analyze any of the ether-coagulated precipitates (total-globulin and β-plus-γ-globulin fractions).

Methods

Dialysis

Wolfson-Cohn fractions were prepared from serum by the method referred to in Part I, and aliquots were pooled to provide sufficient material for electrophoretic analysis. Centrifuged precipitates were drained, dissolved in barbital buffer, and subjected to a preliminary dialysis against barbital buffer to reduce the residual salt concentration and then to a second dialysis against a fresh portion of buffer. Unprecipitated fractions were intermittently dialyzed against running, cold tap water (to remove the bulk of the precipitating salt) and concentrated in a dialysis bag suspended before an electric fan at room temperature. After suitable concentration they were dialyzed against successive portions of barbital buffer as in the case of the precipitated fractions. The final volume was measured so that results could be corrected to the same dilution as that used in the analysis of the whole serum.

Electrophoresis

Electrophoresis was done according to the description in Part I, with the exception that a horizontal slit was substituted for the knife-edge in the scanning procedure. This modification produces a 2-edged pattern. Both edges were traced and the average area for each component was determined. Only descending-boundary averages are reported below, to avoid corrections for protein concentration gradients in the boundary anomaly of the ascending pattern (4).

Results

Albumin Fractions

Each of the 6 albumin fractions analyzed electrophoretically contained not only the nominal component but also a component indistinguishable from α_1-globulin, and traces of what appeared to be mainly parts of the

Table 1. Recovery of Albumin and Nonalbumin Components in Chemical Albumin Fractions, Determined from Three-Times-Enlarged Descending Electrophoretic Boundaries

Case	Planimetric areas (cm.²)				
	Whole serum		Albumin fraction		
	Albumin	α_1-Globulin	Albumin	"α_1-Globulin"	Other globulin
D. F.	15.8	0.7	12.9	0.7	0.4
B. C.	14.7	1.4	12.1	1.4	0.8
M. K.	14.2	1.1	11.3	0.9	0.4
K. B.	13.6	1.9	10.4	1.7	0.5
P. S.	13.1	2.8	11.7	1.9	0.5
S. C.	8.1	1.8	5.5	2.0	0.6

An area of 1 cm.² is equivalent to a biuret-protein concentration of approximately 0.3 Gm./100 ml., although strict proportionality does not hold.

α_2-globulin complex. Invariably the albumin component derived from the Na_2SO_3 solution was smaller than the albumin component of the whole serum, at equivalent dilutions. With one exception, corresponding α_1-globulin peaks were equal or almost equal in area. Individual results are shown in Table 1. Because of the somewhat arbitrary demarcation of components, and the errors of tracing and measuring, an uncertainty of at least ± 0.1 cm.² is to be expected in the planimetric areas.

To check on the possibility of loss of material during concentration and dialysis of the Na_2SO_3 solution, we determined total protein (biuret) in three preparations (D. F., B. C., S. C.) that were analyzed electrophoretically, the samples for protein analysis having been recovered from the bottom section of the electrophoresis cell. The values obtained were 4.78, 4.09, and 2.36 Gm./100 ml. original serum, respectively. The corresponding initial values (i. e., the Wolfson-Cohn albumin values) were 4.88, 4.22, and 2.49 Gm./100 ml. original serum. The apparent recoveries of biuret-reacting materials were thus 98, 97, and 95 per cent, respectively, indicating almost negligible protein losses after the fractionation step; prior losses, if any, could have been caused by precipitation of albumin. The treatment with Na_2SO_3 and ether might also have liberated such nonprotein materials as lipids and pigments, which could have been extracted by the ether or lost by dialysis, the size of the albumin peak thereby having been diminished.

Gamma-Globulin Fraction

In the γ-globulin fractionation we were able to account for more than 90 per cent of the γ-globulin, on the average, but in 5 cases from 10 to 40 per cent of it was found in the supernatant solutions. Components other

Table 2. Recoveries of γ-Globulin and Other Components by Chemical Fractionation, Determined from Three-Times-Enlarged Descending Electrophoretic Boundaries

Case	*Electrophoretic γ-globulin in whole serum*		*Recoveries (% of γ-globulin area of whole serum)*				
			γ-globulin			*Other components in ppt.*	*Apparent γ-glob. total in ppt.*
	Concentration (gm./100 ml.)	*Area* (cm.²)	*Ppt.*	*Supernatant*	*Total*		
J. M.	0.28	1.0	45	40	85	1620	1665
B. C.	0.66	2.5	65	35	100	55	120
S. C.	0.97	3.2	65	30	95	50	115
J. W.	1.47	4.9	90	..	≥90	5	95
H. A.	1.83	6.7	85	20	105	40	125
A. A.	4.87	16.6	75	10	85	15	90

than γ-globulin in the precipitates more than compensated in amount for the γ-globulin that remained in the supernatant solutions. This other material consisted of dissolved protein retained by the unwashed precipitates, and components actually precipitated with the γ-globulin. In one specimen, from a case of β-type multiple myeloma (J. M.), a large part of the abnormal protein appeared in the γ-globulin fraction, although the electrophoretic pattern showed only a trace of γ-globulin. The precipitate from a normal specimen (J. W.) contained 90 per cent of the γ-globulin and a small amount of other material; the corresponding supernatant solution was not examined. Individual results are shown in Table 2, recoveries being expressed to the nearest 5%. Three sets of electrophoretic patterns are shown in Fig. 1.

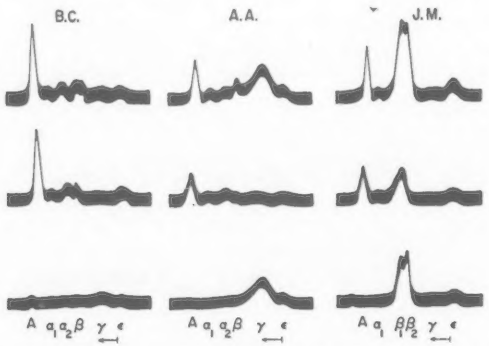

Fig. 1. Descending electrophoretic patterns of whole serum (top row), supernatant of chemical γ-globulin precipitate (middle row), and chemical γ-globulin precipitate (bottom row). Original serum concentrations represented by the patterns are approximately equivalent, with the exception of the γ-globulin precipitate of Patient B. C., which represents approximately twice the concentrations represented by the other patterns.

Less extensive investigations were made of the Na_2SO_4 fractionation procedure, designed to precipitate β- and γ-globulins. In each of three soluble fractions analyzed, a component with β-globulin mobility accompanied the albumin and α-globulin, representing from 6 to 10 per cent of the total electrophoretic areas of the fractions.

III. CALCULATION OF ABSOLUTE ELECTROPHORETIC CONCENTRATIONS

The usual method of calculating absolute concentrations of serum-protein components from a knowledge of their free electrophoretic distribution and of total protein neglects variations in the ratio of protein to nonprotein material among the components. Although the possibility of errors arising from this neglect has been recognized and not infrequently pointed out since the first application of free electrophoresis to serum-protein analysis, it appears to deserve particular emphasis in the present situation. The demonstration of errors with respect to the individual fractions is a complex procedure, but the varying magnitude of the ratio of protein to nonprotein material in whole serum, which underlies the problem, may be easily shown by parallel determinations of total solids retained by a dialysis membrane, and of total protein. An experiment of this kind is described below.

Methods

Total solids retained by a dialysis membrane (TS) were estimated by this procedure: A measured volume of serum (usually 1.00 ml.) was pipetted into a dialysis bag and washed down with 5 ml. distilled water. A blank determination was set up by placing an equal volume of distilled water in another dialysis bag. The bags were tied off, suspended in approximately 500 ml. distilled water contained in a flask, and kept in a refrigerator for 7–10 days with once or twice daily changes of water. At the end of the dialysis period the contents of the bags were emptied quantitatively into respective 30-ml. beakers that had been previously heated to 80–85°, cooled, and balanced against each other on an analytical balance. Numerous small portions of distilled water were used to suspend precipitated protein and wash it from the sample bag. The blank bag was washed in the same way to give the same total volume of water in its beaker.

The two beakers were placed in an oven at 80–85° overnight. The next morning the beakers were cooled and again balanced against each other. The weight change required to achieve a balance was recorded as the first estimate of TS. The oven-drying of the residues was then resumed, with

additional weighings at intervals of approximately 8, 24, 48, and sometimes 72 hours after the first weighing. The residue weight usually passed through a minimal value between the first and last weighings. The lowest value was taken to be the best estimate of the TS of the serum sample. The blank values, which were automatically subtracted by the method of weighing used, were of the order of 1 mg.

Serum specimens from 22 individuals, representing a wide variety of protein patterns, were analyzed for TS. A total-protein value (TP), by the method referred to in Part I, and an electrophoretic pattern, by the method described in Part II, were also obtained for each specimen. The sera were diluted equally for electrophoretic analysis and slight volume differences that developed during dialysis were neglected. Total electrophoretic areas were determined from the 3-times-enlarged descending patterns, exclusive of the boundary anomaly.

Results

The respective relationships between TS and TP and the electrophoretic area are shown in Fig. 2. The linear regression lines, calculated by the method of least squares, are drawn in. The plot confirms the expected proportionality between TS and the area, the value of TS corresponding to zero area (−0.078 Gm./100 ml.) being not significantly different from

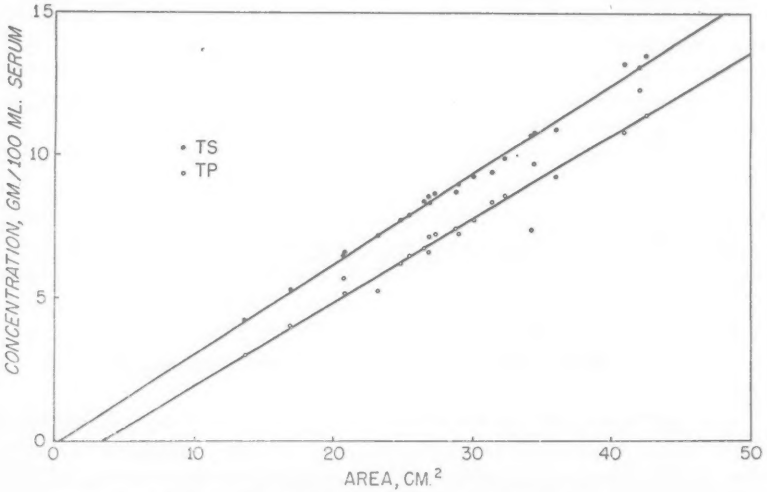

Fig. 2. Plots of TS and TP vs. electrophoretic area.

zero ($p > 0.5$). The value of the TP-intercept (-0.976 Gm./100 ml.) was found to be significantly less than zero ($p < 0.02$).

The standard deviation of the TS values about their regression line was 0.183 Gm./100 ml., or 2.0 per cent at the mean value, 8.99 Gm./100 ml. Some of this variation must be attributed to experimental errors and inadequacies. If the desired quantities could be measured with perfect accuracy, the residual variation presumably would be determined by refractive-index-increment variations. One would expect this effect to be small, judging from the results of Armstrong *et al.* (5), who found that the refractive-index increments of unfractioned proteins from normal and pathologic plasma specimens did not differ from the mean value for normal plasma proteins by more than 5 per cent.

The standard deviation of the TP values about their regression line was 0.494 Gm./100 ml., or 6.6 per cent at the mean value, 7.44 Gm./100 ml. This relatively large variation was undoubtedly caused by deviations of the nonprotein fraction from the general trend: an absolute increase but a relative decrease with increasing values of TP. The values of the ratio TP/TS ranged from 0.69 to 0.94. The serum with the lowest value was obtained from a patient with hypercholesterolemic xanthomatosis and that with the highest value, from a patient with Waldenström's macroglobulinemia syndrome. Specimens from 3 healthy individuals included in the series had values from 0.81 to 0.86, but several specimens with abnormal electrophoretic patterns also fell in this interval. Even those TP values corresponding to the highest values of TP/TS failed to be included in the range of variation of the TS values about their regression line.

DISCUSSION

A fair comparison between the results of chemical and free electrophoretic determinations of serum proteins would require measurement of the same material in purportedly corresponding fractions. When, as in the present instance, the chemical fractions are analyzed only for the simple-protein constituent, the nonprotein materials (lipids, carbohydrates, pigments, etc.) bound to the protein, and hence measured electrophoretically, are largely neglected. One should then expect absolute agreement only in the limiting case in which nonprotein material is absent. In other cases electrophoretic areas representing nonprotein material are, by the usual method of calculation, assigned protein concentrations at the expense of the rest of the pattern, unless the ratio of protein to nonprotein material happens to be constant throughout the

pattern. Since the occurrence of a constant ratio is highly improbable, electrophoretic concentrations calculated from simple-protein totals will tend to become less reliable as the fraction of nonprotein material increases. Usually this fraction is greater than 10 per cent and in some cases may exceed 30 per cent. If such a large fraction is concentrated in one component, for example, or in all but one component, concentrations calculated from the simple-protein total will be subject to errors of considerable magnitude. To put the comparison between the Wolfson-Cohn and electrophoretic methods on a common basis one would have to determine protein factors for the various electrophoretic components or measure the total solids (retained by a dialysis membrane) in the chemical fractions.

Phosphate-Turbidity Method

Quite recently Aull and McCord (6) devised a phosphate-turbidity method for estimating serum-protein fractions, and compared results obtained by their method with those obtained by the Wolfson-Cohn method. They found good correlations in the albumin and γ-globulin determinations, but not in the α- and β-globulin determinations. They suggest that their method may measure certain nonprotein materials not measured by the Wolfson-Cohn method, and that therefore results obtained by the phosphate-turbidity method in the determination of α- and β-globulins may be found to be more closely correlated with electrophoretic than with the chemical results. This suggestion is supported by their scatter diagrams of the total-globulin and α-globulin values, but not by the scatter diagram of the β-globulin values, the latter showing no general trend toward higher values by phosphate turbidity. Moreover, if the observed differences between the two methods are caused mainly by measurement of different materials, the chemical method must measure a significant amount of material not measured by phosphate turbidity, because the total-protein values are shown to be in good average numerical agreement.

Reasons for Discrepancy between Chemical and Electrophoretic Methods

Part of the true discrepancy between the Wolfson-Cohn and free electrophoretic fractionations almost certainly may be attributed to faulty fractionation by the chemical method. If, as the electrophoretic evidence suggests, varying amounts of α-globulin are included in the albumin fraction, varying amounts of β-globulin are included in the albumin-plus-α-globulin and in the γ-globulin fractions, and varying amounts of γ-glob-

ulin fail to be precipitated in the γ-globulin fraction, the determinations of α- and β-globulins especially, by calculation of differences between appropriate quantities, would obviously be erratic. The fact that the chemical β-globulin values were as a rule higher than the corresponding electrophoretic values could be taken as further evidence of faulty fractionation, because one would expect the difference to be in the opposite direction, owing to the relatively large amount of nonprotein material that is usually in the β-globulin fraction. Finally, the chemical α-globulin values agreed much better with the electrophoretic α_2-globulin values than with the electrophoretic total-α-globulin values. Good though it may be, this numerical agreement should not be considered evidence that the Wolfson-Cohn Na_2SO_3 and Na_2SO_4 treatments sharply define α_2-globulin on a solubility basis; the electrophoretic evidence for overlapping distributions is too strong.

Evaluation of Wolfson-Cohn Method

Insofar as the clinical application of the Wolfson-Cohn procedure is concerned, the determination of albumin seems sufficiently reliable to be recommended for routine use; serious consequences as a result of the apparent contamination of the albumin fraction with α_1-globulin seem unlikely. The γ-globulin values should perhaps be viewed more cautiously, for here, in spite of good correlation with electrophoretic values, the numerical agreement is relatively poorer than in the albumin determination and appears to depend to an appreciable extent on compensation of fractionation errors, and in rare cases a completely erroneous impression of the γ-globulin concentration may be obtained. Nevertheless, a high chemical γ-globulin value would almost certainly indicate an increased amount of protein somewhere in the globulin fraction. If one could be sure that the Wolfson-Cohn γ-globulin procedure would rarely if ever give values in or above the normal range when the γ-globulin level was actually low, it would be a valuable screening procedure for the detection of agammaglobulinemia or hypogammaglobulinemia. The Wolfson-Cohn α- and β-globulin values, being subject to the errors of two and three other determinations, respectively, must be regarded as rough approximations at best.

CONCLUSIONS

When one considers, in addition to the uncertainties already discussed, the errors associated with quantitative interpretation (4, 7, 8) and planimetry of the electrophoretic pattern, and with pipetting, mixing, and

colorimetry in the Wolfson-Cohn procedure, the surprising thing is not the lack of agreement with respect to the determinations of α- and β-globulins, but the generally good agreement with respect to the determinations of albumin and γ-globulin. Although further improvements will certainly be forthcoming, it is doubtful that a small group of simple chemical fractionations will ever be devised to handle adequately all possible serum-protein derangements.

SUMMARY

One hundred serum specimens from healthy and diseased adults were analyzed for the major protein constituents by free electrophoresis and by the Wolfson-Cohn chemical method. Albumin values obtained by the two methods were well correlated and were also in good average numerical agreement. The γ-globulin values were well correlated, but were not in quite such good numerical agreement, on a percentage basis, as were the albumin values. The α-globulin values were poorly correlated, as were also the β-globulin values.

Electrophoretic examination of some of the chemical fractions indicated that part of the discrepancy between the two methods was caused by faulty chemical fractionation, particularly with respect to the globulin fractions.

Determination of total solids retained by a dialysis membrane (TS) in 22 serum specimens confirmed the proportionality between TS and the electrophoretic-pattern area, and emphasized a source of error of undetermined magnitude in the calculation of electrophoretic results from simple-protein totals.

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Note on the Identification of Alkaloids by Paper Chromatography

Guy Nadeau

In 1949, Munier and Macheboeuf (1) suggested the use of ascending paper chromatography for the isolation and identification of various alkaloids and various organic nitrogen compounds. Their procedure was most successfully applied by Mannering and his associates (2) to the detection of opium alkaloids in urine and tissues. The conditions set by the latter workers led to such interesting results in our hands that they were used as the basis of the present study. From the beginning, however, it was felt that the apparatus, as well as the technic, could be somewhat simplified.

MATERIALS AND METHODS

The alkaloids selected were atropine, cocaine, codeine, morphine, dionine, dilaudid (dihydromorphinone), heroin, strychnine, meperidine and papaverine. After the alkaloids (100 μg. in a 2.5% solution of appropriate solvent) had been applied at the bottom of rectangular sheets of filter paper, they were chromatographed in a container made from a conventional aquarium covered with a heavy glass plate. Since variations in temperature will affect R_f values, a thermometer was left in the container. The sheets were rolled into a cylinder and the edges were held with staples. After rise of the solvent to about 2 cm. from the upper edge, the paper was dried at room temperature with the aid of an electric fan. The sheets were then either sprayed with the reagent by use of an ordinary atomizer or soaked in the reagent solution itself.

Of the solvents investigated, *n*-butanol or isobutanol, saturated with

From the Laboratoire de Biochimie, Hôpital Saint-Michel-Archange, Mastai, Québec, Canada.

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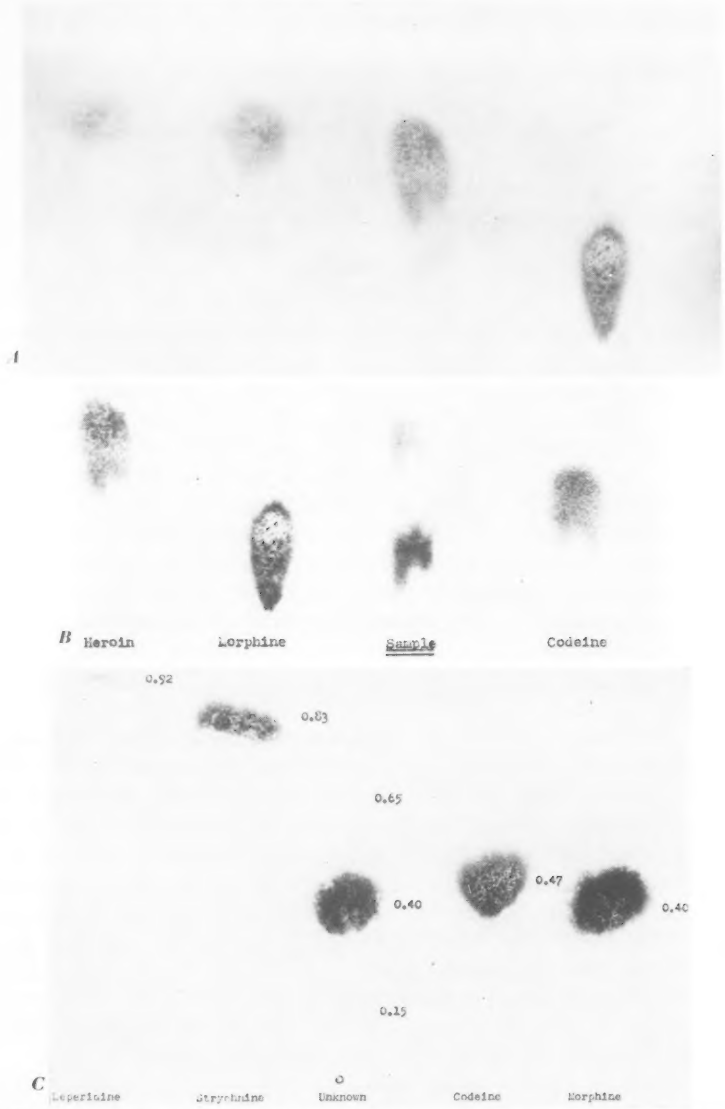


Plate 1. *A*, Distribution of various alkaloids with isobutanolacetic acid, following saturation of paper with aqueous phase of solvent. *B*, The powdered sample was supposedly pure heroin. Result showed that sample (third spot from left) was a mixture of heroin and morphine (first and second spots). *C*, Unknown sample (spot in center) revealed presence of morphine (identical R_f and identical coloration with potassium iodoplatinate).

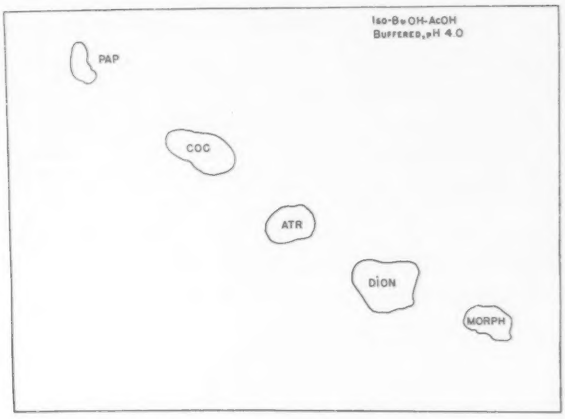


Fig. 1. Distribution of various alkaloids on acid-buffered paper.

an aqueous solution of acetic acid (alcohol, 10 vol.; glacial acetic acid, 1 vol.; and water, 5 vol., shaken several times throughout a 3-day period, then decanted) gave most satisfactory results, the spots being very definite in shape and distributed over a wide range (Plate 1, *A*, and Fig. 1).

Whatman No. 1 and No. 4 filter papers, 22 × 28 cm., were found satisfactory. Spots were somewhat more definite in shape with No. 1 paper. No. 4, being less retensive, allowed faster rise of the solvent—in from 6 to 7 hours—as compared with 16 hours with No. 1 paper. The latter was, however, found more convenient for overnight operation.

Spraying with dilute potassium iodoplatinate[1] (2) was found most convenient. Five μg. of morphine were clearly discernible with this reagent. Dragendorff's reagent[2] (1, 2) yielded comparable results, but the first reagent was preferred, since the color of the spots provided further information on the nature of the alkaloid. Identification by ultraviolet light, being nonspecific for alkaloids, was discarded.

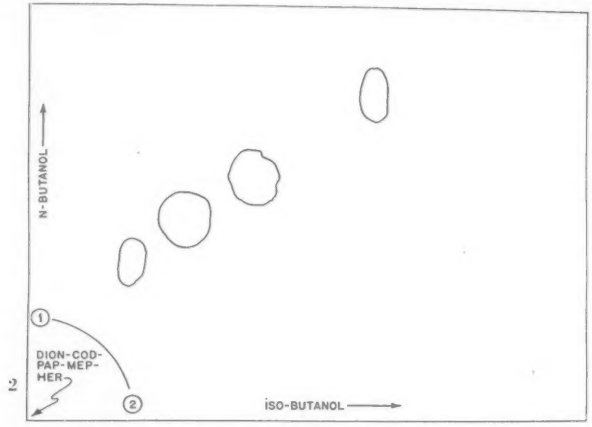
Proper presaturation of the paper is essential in order to avoid trailing of the spots. Best conditions of saturation were obtained by leaving the paper in an atmosphere saturated with the aqueous phase of the solvent for 2 to 3 hours before dipping it in the solvent itself.

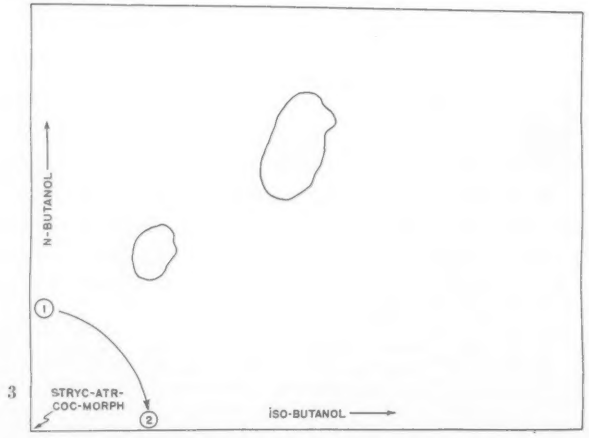
[1] Potassium iodoplatinate reagent: Dissolve 1 Gm. of platinic chloride in 10 ml. of water, mix with 250 ml. of 4% potassium iodide, and add water to 500 ml.

[2] Dragendorff's reagent: Mix together 2.5 Gm. of bismuth subnitrate, 10 ml. of 40% potassium iodide, 5 ml. of glacial acetic acid, and 20 ml. of water. The precipitate that forms on standing is removed by filtration. Just before using, mix one part of the filtrate with two parts of glacial acetic acid and three parts of water.

RESULTS

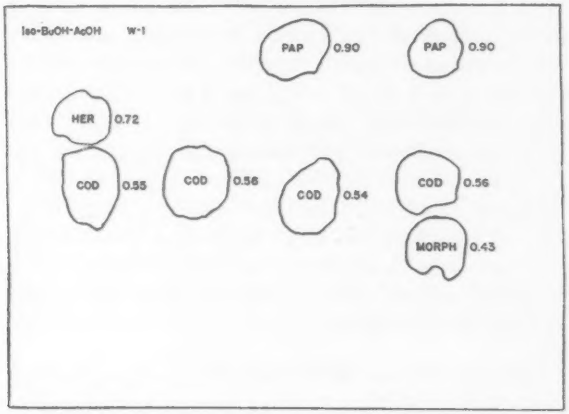
R_f values were reproducible for every one of the alkaloids selected. Morphine and dilaudid reached about the same level, the lowest in the series, but identification was made possible by the different coloration given with the iodoplatinate reagent: intense blue with morphine, violet with dilaudid. Meperidine and papaverine both rose to the highest level in the series, but the definite lilac tint of papaverine was easily distinguishable from that of meperidine (bluish violet). The other alkaloids were distributed between these two pairs.

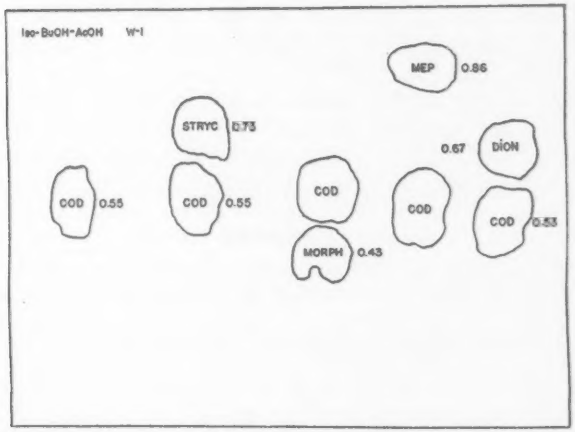




Figs. 2 and 3. Two dimensional chromatograms (buffered paper).

Alkaloids are more sharply resolved on paper which has been previously moistened with acid buffer solutions (2, 3). The paper is usually dipped in the buffer and the excess removed by pressing between porous paper. The solutions to be assayed are then applied on moist paper. This was found most inconvenient, first because the solution has to be dried after each deposit, second because of the difficulty of fashioning the wet paper into consistent cylinders. In our case, solutions of the alkaloids were applied at the bottom of the dry paper which was afterwards rolled into a cylinder, then evenly sprayed with a citrate-phosphate solution





Figs. 4 and 5. Distribution of mixtures of various alkaloids with codeine (unbuffered paper).

buffered at *p*H 4.0. Papers were chromatographed at once. Due to the competitiveness of this nonmobile phase, the rise of the solvent was much slower (from 21 to 24 hours), but there was indeed a wider and more definite distribution of spots (see Plate 1, *A*, and Fig. 1).

No advantage was found in performing two-dimensional chromatograms in the conditions of the present investigation. Some alkaloids were constantly superposed (Figs. 2 and 3). This, however, might have been avoided by a more discriminate choice of solvents.

BIOLOGIC APPLICATION

Possible application of the method to biologic material was emphasized by the following simplified extraction procedure which allowed the detection of less than 5 μg. of morphine. Two hundred and fifty micrograms of the alkaloid were added to 25 ml. of urine. This was made alkaline to phenolphthalein with ammonium hydroxide and extracted once with 5 ml. of isoamyl alcohol. One fiftieth of this volume was chromatographed. In actual determinations, of course, urine would first be acidified and autoclaved to effect hydrolysis of the conjugated form. Codeine was also easily detected, by the same procedure, in the morning urine of a control subject who had, the evening before, taken a tablet containing 8 mg. of the alkaloid.

IDENTIFICATION

In order to eliminate errors of identification, especially if exactly identical conditions of operation are not adhered to, the best policy (and our adopted procedure) is to spot a sample of the suspected alkaloid along with the unknown at the bottom of the same sheet of filter paper (Plate 1, *B* and *C*). Another means of avoiding false interpretations, is to add traces of the suspected alkaloid to the solution to be assayed. The formation of two distinctive spots when the sample solution itself had given only one, rules out the identity of alkaloids (Figs. 4 and 5).

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Simplified Rapid Technic for the Extraction and Determination of Serum Cholesterol without Saponification

Julius J. Carr and I. J. Drekter

THE VALUE OF CHOLESTEROL DETERMINATIONS as an aid to diagnosis and treatment in a variety of clinical states, such as arteriosclerosis, biliary obstruction, diabetes, liver disease, and nephrosis, is well recognized. However, clinical laboratories are often unable to keep pace with the growing demand for these determinations because of the laborious and exacting nature of the more reliable technics (19, 23, 24). The need existed, therefore, for a simple, rapid, yet accurate technic which could readily accommodate a fairly large number of specimens in a single series.

With a few exceptions (10, 12, 25, 27, 30), colorimetric methods for the determination of cholesterol are based upon the original reaction of Liebermann and Burchard, although they often differ in the method of extraction. Solvent extraction from serum dried on plaster of paris (11) or paper (9, 20) is cumbersome and tedious. Wet-extraction methods employing such solvents as ethanol-ether (2, 3, 5, 7, 16, 28), ethanol-acetone (19, 22–24), and petroleum ether (1, 27) require at least two additional steps for analysis: removal of solvent and its replacement by a medium suitable for color development. Direct extraction by chloroform (8, 31) achieves a considerable simplification in technic. However, ample evidence (5, 7, 8, 15, 23, 26, 29) exists in support of Bloor's (3) finding that cholesterol esters produce greater color intensities than does an equivalent concentration of free cholesterol, when the Liebermann-Burchard reaction is carried out *in chloroform*. Values for total cholesterol are, therefore, incorrect when the Liebermann-Burchard reaction is applied, without prior saponification, to extracts of serum cholesterol

From the Department of Chemistry, The Mount Sinai Hospital, and the Drekter-Heisler Medical Laboratories, New York, N. Y.

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in chloroform. Application of a factor (7, 8, 26) to correct for these differences in color intensity is strictly valid only when an analysis for free cholesterol is also performed, as in the method of Teeri, since it can be shown that a correct factor necessarily varies with the ratio of free to total cholesterol. It has been found by the authors that extraction of serum cholesterol into acetic anhydride, as proposed by Drekter, overcomes these difficulties. We have been able to show that in an acetic anhydride–acetic acid solvent *equal* maximum color intensities are produced by equimolar concentrations of free and esterified cholesterol, upon treatment with a modified Liebermann-Burchard reagent. A careful survey of the literature has revealed no experimental refutation of this finding.

A method (13) similar to ours has appeared while this work was in progress. The method is quite rapid and convenient, but we find that when color is developed in the presence of serum proteins, as recommended by Pearson, Stern, and McGavack, the values obtained for cholesterol are approximately 10 per cent too low. A second disadvantage is the possibility of explosive reactions (6), which may result from accidental introduction of an excessive quantity of water, when the hygroscopic reagent *p*-toluenesulfonic acid is used. Finally, in view of the known sensitivity of the Liebermann-Burchard reaction to changes in temperature (5, 8, 18, 19), closer control of temperature is indicated.

The procedure detailed below, embodying extensive modifications of a direct extraction method with acetic anhydride (4), should fulfill the stated need for a simple, rapid, yet thoroughly reliable method for the determination of cholesterol in serum or plasma. In our laboratories this method has given consistently satisfactory performance in more than 13,000 analyses during a 3-year period. Equally good results have been reported to us by other laboratories which have adopted the procedure.

MATERIALS AND METHOD

Reagents

Glacial acetic acid, reagent grade.

Acetic anhydride, reagent grade. Keep tightly stoppered.

Concentrated sulfuric acid, reagent grade. Keep tightly stoppered.

Cholesterol standard, 200 mg. per 100 ml.: Dissolve 0.2000 Gm. recrystallized cholesterol, m.p. 148°, in approximately 50 ml. glacial acetic acid. Dilute with glacial acetic acid to exactly 100 ml.

Sulfuric acid–acetic acid reagent, 1:1 (v/v): Slowly pour 100 ml. conc. H_2SO_4 into a 500-ml. Pyrex flask containing 100 ml. glacial acetic acid.

Mix the contents by gentle rotation until the addition has been completed. Allow the mixture to cool to room temperature before using. This reagent is stable at room temperature for at least 6 months. Keep well stoppered.

Dehydrating reagent: Mix 10 ml. H_2SO_4-acetic acid reagent (1:1) with an equal volume of glacial acetic acid. Transfer to a dropping bottle. The reagent is stable for at least 6 months.

Instrumentation: Satisfactory results have been obtained with the Klett-Summerson photoelectric colorimeter and with the Junior and Universal models of the Coleman spectrophotometer. Other photometers capable of measuring absorbence at 620 mμ are probably satisfactory.

Procedure

Test Sample

Accurately pipet 0.2 ml. serum into a test tube suitable for centrifugation. Thick-walled, lipless tubes, measuring 125 x 16 mm. are recommended. Add 0.8 ml. glacial acetic acid to the serum, mix gently and allow to stand for 1–2 minutes.

Serum Blank

Repeat the above procedure using a second 0.2-ml. aliquot of serum.

Reagent Blank

Add 0.8 ml. glacial acetic acid to 0.2 ml. distilled water.

Standard

Measure 0.2 ml. cholesterol standard into a fourth tube. Add 0.6 ml. glacial acetic acid and 0.2 ml. distilled water. Prepare this standard for each series of determinations.

Calibration

To calibrate a photometer for this procedure, prepare a series of standards by measuring 0.1, 0.2, 0.3, 0.4, and 0.5 ml. cholesterol standard into separate tubes. Add glacial acetic acid to a final volume of 0.8 ml. Finally, add 0.2 ml. distilled water to each and mix gently. For highest accuracy duplicate standards, accompanying each set of determinations, are more reliable than an occasionally prepared calibration curve.

Extraction and Deproteinization

To each sample, prepared according to the procedures outlined above, add 4 ml. acetic anhydride. Adequate mixing is achieved by allowing the

reagent to flow freely into each sample without contacting the wall of the tube. If necessary, complete the mixing process by imparting a rotary motion to the contents of the tube. Precipitation of serum proteins commences after approximately 2 ml. acetic anhydride have been added. Centrifuge all serum-containing samples for 5 minutes at approximately 2000 rpm. By decantation, transfer the clear supernatant solution as completely as possible into a similar tube. Discard the protein precipitate. Add 1 ml. glacial acetic acid to each serum blank only, and set it aside for photometric reading.

Dehydration and Development of Color

Allow 1 drop of dehydrating reagent to fall directly into each solution other than the serum blank. Mix by rotation. This step is accompanied by a marked increase in temperature after a brief induction period. If the solution does not become hot within 1–2 minutes, repeat with not more than 1 additional drop of reagent. Immediately after dehydration, transfer each specimen to a water bath regulated at 25°. A rectangular enameled pot with a capacity of approximately 6 L. is most inexpensive, yet wholly suitable. When the vessel is half-filled with water at 25°, the temperature is usually maintained within satisfactory limits, *i.e.*, 1°, for at least 1 hour without further regulation.

After the contents of the tubes have reached bath temperature (approximately 5–10 minutes), pipet 1 ml. of H_2SO_4-acetic acid reagent into the tube containing the reagent blank. The reagent should drop directly into the solution without touching the wall of the tube. Promptly remove this tube from the water bath and ensure complete mixing by holding the upper portion of the tube firmly in the left hand while imparting a rotary motion to the contents by tapping the bottom of the tube with the fingers of the right hand. Replace at once in the water bath. Do not permit water to drop into tubes by accidental splashing. With a little practice the addition of reagent and mixing can be completed within ½ minute. At successive 1-minute intervals following the addition of H_2SO_4-acetic acid reagent to the reagent blank, repeat this process with the standard and with the test sample. By maintaining exact intervals during color development, followed by the same intervals during readings of absorbence, equal periods of color development are assured for all specimens, even when many samples are analyzed in series.

Transfer the reagent blank to a suitable photometer cuvet or cell. Twenty minutes after the addition of H_2SO_4-acetic acid reagent adjust the photometer to read zero absorbence (or Klett units) against the reagent blank at 620 mμ (Klett filter No. 62). Read the standard and test

specimens at successive 1-minute intervals. Finally, read the absorbence value of the serum blank. After measurement of their absorbences, reaction mixtures are disposed of by pouring into a large amount of water. Do not add water to tubes containing these mixtures.

Calculation

When photometers which show conformity to Beer's law for this color reaction are employed (*e.g.*, Coleman or Klett), the average absorbence of duplicate standards is used for the following calculation:

$$mg.\ total\ cholesterol\ per\ 100\ ml.\ serum = \frac{D_T - D_B}{D_S} \times C_S \times \frac{100}{0.2} \times 1.01^1$$

where D_T = absorbence of test specimen
D_B = absorbence of serum blank
D_S = average absorbence of duplicate standards
C_S = amount of cholesterol (0.4 mg.) in 0.2 ml. of standard

For a given value of D_S, the expression may be reduced to

$$K(D_T - D_B)$$

where $K = \frac{202}{D_S}$

EXPERIMENTAL

Optimum Conditions for Color Development

A 0.4 ml. amount of a solution containing 200 mg. of cholesterol in 100 ml. of glacial acetic acid was pipetted into a Klett cuvet. To this was added 0.4 ml. glacial acetic acid, 0.2 ml. distilled water, and 4 ml. acetic anhydride. Following the addition of 1 drop of dehydrating reagent, the temperature rose spontaneously to 50–60° after a short induction period, usually a few seconds. After the mixture was cooled to 25° in a water bath, 1 ml. of 25 parts by volume of H_2SO_4 in 75 parts by volume of glacial acetic acid was introduced. The contents of the tube were promptly mixed and the tube then returned to the water bath. A Klett photoelectric colorimeter was adjusted to zero reading at 620 mμ (Klett filter No. 62) against a reagent blank. Periodic readings of color intensity were then recorded at fixed intervals.

Similar studies were conducted by employing 1 ml. of each of the following mixtures as the color-developing agent: 50% H_2SO_4 in acetic acid (v/v), 75% H_2SO_4 in acetic acid (v/v), and 98–99% H_2SO_4 (concen-

[1]Application of the factor 1.01 is discussed in a later section. The factor is valid when the extract is decanted as completely as possible from 125 × 16 mm. tubes.

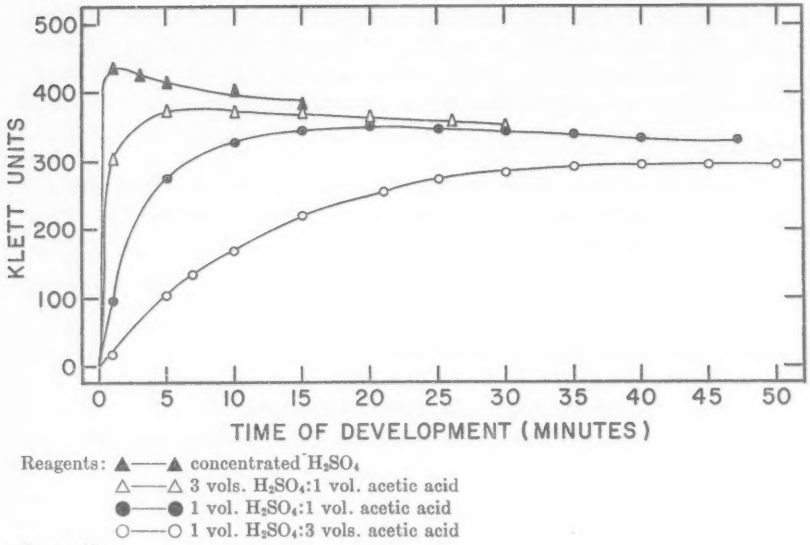
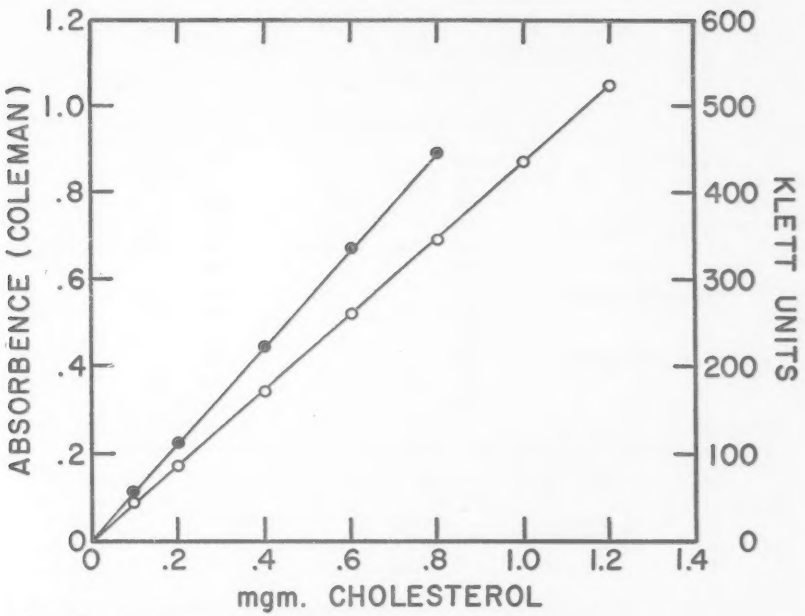


Fig. 1. Rate of color development as a function of sulfuric acid concentration

trated). The variation of color intensity with time of development is shown for each reagent in Fig. 1. Although maximum color intensity is a function of H_2SO_4 concentration, it is evident, too, that color stability varies inversely with the concentration of this reagent. It can also be seen that the time required for development of maximum color intensity decreases with increasing concentration of H_2SO_4. A reagent consisting of equal volumes of H_2SO_4 and acetic acid possesses the advantages of satisfactory sensitivity, adequate color stability, and a reasonable period for development of color (20 minutes). A maximum of 1 per cent variation in color intensity is observed during the 15–25 minute interval following addition of the reagent.

Relation between Cholesterol Concentration and Absorbence

Varying volumes of a standard solution containing 2 mg. cholesterol per ml. glacial acetic acid were measured into tubes. All volumes were adjusted to 0.8 ml. by the addition of glacial acetic acid. Following the addition of 0.2 ml. of water to each tube, each determination was completed in the usual manner. It is evident from Fig. 2 that Beer's law is closely obeyed at 620 mμ, within the useful operating ranges of the Klett



●——● Coleman Junior spectrophotometer, 620 mμ, 19-mm. cuvet
○——○ Klett-Summerson photoelectric colorimeter, filter no. 62, 14-mm. cuvet
Final volume of solution 6.0 ml., temp. 25°

Fig. 2. Variation of color intensity with cholesterol concentration

photoelectric colorimeter and of the Coleman Junior spectrophotometer. These curves have been found to be closely reproducible for a given instrument. Although the use of these curves for the calibration of other instruments of the same manufacture is not recommended, they may serve as a guide to expected readings.

Relative Chromogenicity of Free Cholesterol and Cholesterol Esters

One hundred milliliters of 5.17×10^{-3} *M* solutions of recrystallized preparations of cholesterol (m.p. 148.5°), cholesteryl acetate (m.p. 113.5°), cholesteryl stearate (m.p. 79.5°), and cholesteryl oleate (m.p. 39°) were prepared by dissolving 200.0, 221.8, 337.8, and 336.8 mg., respectively, of these substances in 100 ml. of chloroform. Two-tenths ml. of each solution was treated successively with 0.8 ml. glacial acetic acid, 0.2 ml. water, and 4.0 ml. acetic anhydride. Each analysis was then completed in the usual manner. Samples of the same sterol solutions

Table 1. Comparison of Readings Obtained with Cholesterol and Cholesterol Esters (Klett, Filter 62)

Substance	Proposed method			Bloor method		
	Reading	Average	Difference, %	Reading	Average	Difference, %
Cholesterol	199			241		
	202	201	..	239	239	..
	202			238		
Cholesteryl acetate	198			268		
	202	200	−0.5	268	268	+12.1
	200			268		
Cholesteryl stearate	204			302		
	202	204	+1.5	303	302	+26.2
	205			301		
Cholesteryl oleate	196			266		
	197	197	−2.0	269	268	+12.1
	197			269		

Table 2. Recovery of Cholesterol Added to 0.2 ml. Serum

Sample	Present (mg.)	Added (mg.)	Found (mg.)	Recovery (%)
1	0.326	0.200	0.530	100.8
2	0.326	0.200	0.524	99.6
3	0.326	0.400	0.716	98.6
4	0.326	0.400	0.728	100.3

were also treated in chloroform with Liebermann-Burchard reagent according to a modification (14) of the original Bloor technic (2).

From the results shown in Table 1, it can be seen that equivalent concentrations of free cholesterol, cholesteryl acetate, cholesteryl stearate, and cholesteryl oleate produce essentially equal color intensities when the technic described by the authors is followed. On the other hand, when color development is carried out in chloroform, the esters of cholesterol produce considerably more color than does the free alcohol.

Recovery of Cholesterol Added to Serum

To individual 0.2-ml. aliquots of a serum sample were added 0, 0.1, and 0.2 ml. of a standard solution containing 0.2 mg. of cholesterol per 0.1 ml. of acetic acid. Following adjustment of the total volume to 1 ml. by the addition of glacial acetic acid, each sample was analyzed for cholesterol in the usual manner. Satisfactory recovery of added cholesterol is shown in Table 2.

Effect of Illumination on Color Development

Five samples of serum were analyzed for cholesterol by the procedure adopted by the authors. During color development, illumination was

Table 3. Comparison of Cholesterol Values Obtained after Development of Color in the Dark and Under Strong Illumination

Specimen	Cholesterol found (mg. per 100 ml.)		Deviation, %
	Illuminated development	Dark development	
106	260	258	+0.8
	260	264	−1.5
111	223	231	−3.5
	220	225	−2.2
122	151	154	−1.9
	153	152	+0.7
139	278	272	+2.2
	278	278	0.0
170	290	292	−0.7
	290	295	−1.7
Average	240	242	±1.5

Table 4. Serum Cholesterol Values as Obtained by the Proposed Method and by the Schoenheimer-Sperry Method

Serum no.	Mg. cholesterol per 100 ml. serum		Deviation (%)	Serum no.	Mg. cholesterol per 100 ml. serum		Deviation (%)
	Carr-Drekter	Schoenheimer-Sperry			Carr-Drekter	Schoenheimer-Sperry	
775	115	109	+5.5	728	241	242	−0.4
101	150	143	+4.9	95	241	253	−4.7
783	152	158	−3.8	196	250	257	−2.7
723	153	154	−0.7	700	262	261	+0.4
709	170	181	−6.1	718	269	269	0.0
159	176	184	−4.3	736	269	277	−2.9
730	177	171	+3.5	83	280	287	−2.4
193	179	178	+0.6	96	284	294	−3.3
999	183	186	−1.6	756	291	278	+4.7
757	208	198	+5.0	744	316	312	+1.3
162	212	219	−3.2	90	328	319	+2.8
743	221	233	−5.1	751	336	318	+5.7
722	232	236	−1.7				
Average					228	228	±3.1

provided by diffused daylight reinforced by 1120 watts of fluorescent room lighting at a distance of 4 to 8 feet. In a duplicate series the Liebermann-Burchard color was developed in a dark cabinet. Comparative results, as summarized in Table 3, show that even relatively strong lighting exerts no appreciable effect on color intensity.

Comparison of Proposed Method with Schoenheimer-Sperry (19) Procedure

Twenty-five randomly selected samples of serum, obtained from hospitalized patients, were analyzed for cholesterol according to the proposed technic, as well as by the method of Schoenheimer and Sperry. The averages of duplicate values, listed in Table 4, show good agreement between both methods. No trend toward higher or lower values is shown by either procedure. Ten values were higher than the corresponding Schoenheimer-Sperry results by an average of 3.4 per cent; fourteen values were correspondingly lower by an average of 3.1 per cent. The degree of correspondence of values for individual samples, as obtained by each method, as well as the coincidence of the average for each group (228 mg. per 100 ml.), supports the validity of the proposed method. The agreement of results also demonstrates that extraction of serum cholesterol is complete and that both methods are equally specific for cholesterol. The results further confirm the reported finding of equal chromogenicity of free and esterified cholesterol.

DISCUSSION

Functions of Reagents Employed

Although purified free cholesterol and its esters are soluble in the usual lipid solvents (*i.e.*, petroleum ether, diethyl ether, chloroform, etc.), these solvents are not suitable for the direct extraction of esterified and so-called free cholesterol from serum. Both forms must first be freed from their associated lipoproteins before extraction can be effected. Mixtures of ethanol and ether (2, 3, 5, 7, 16, 28) and of ethanol and acetone (19, 23, 24) are effective extracting agents, but these solvents interfere with the Liebermann-Burchard reaction. For example, ethanol, in the presence of H_2SO_4, adversely effects the determination by removing a considerable portion of essential acetic anhydride through esterification. These solvents, when present, must therefore be removed before color development. On the other hand, glacial acetic acid is well suited to a direct technic for cholesterol, since it facilitates quantitative extraction of serum cholesterol, yet it does not interfere with the Liebermann-Burchard reaction. A 4:1 ratio of glacial acetic acid to serum suffices for complete extraction. Lower ratios may result in incomplete extraction and may also cause the mixture to gel. No significant extraction of cholesterol is effected by acetic anhydride alone.

Acetic anhydride, in the presence of acetic acid, fulfills the multiple functions of cholesterol extraction, precipitation of serum protein, and

furnishing a medium suitable for color development. Acetic anhydride also facilitates production of the necessary anhydrous system before addition of color reagent (4). Because the intensity of color produced is a function of the concentration of acetic anhydride, adequate control of this concentration as well as of the total water content is essential.

The deleterious effect on the Liebermann-Burchard reaction of relatively small quantities of water is well known (5, 15, 18). The effect is due to the presence in the same system of two mutually reactive substances: water and acetic anhydride. The addition of a strong acid, as H_2SO_4 in the Liebermann-Burchard reagent, to such a mixture would serve to catalyze the strongly exothermic hydrolysis of acetic anhydride. The temperature would rise so rapidly that its adequate control, an essential condition for reproducibility of absorbence readings (5, 7, 18, 19, 22), could not be maintained. It is therefore necessary to effect dehydration before color development is begun. This may be accomplished by allowing the extract to stand overnight at room temperature, by heating the extract for 30 minutes in a boiling water bath (4), or most simply by means of the dehydrating reagent. The quantity of H_2SO_4 chosen for this purpose is sufficient to catalyze the reaction between water and acetic anhydride, yet it is not adequate for the initiation of color development. After dehydration, a sufficient excess of acetic anhydride is available for the Liebermann-Burchard reaction.

The H_2SO_4-acetic acid reagent is basically a modified Liebermann-Burchard reagent. It is designed to provide adequate sensitivity and stability of color for the conditions of analysis adopted by the authors. Compared with mixed H_2SO_4-acetic anhydride reagents, it possesses important advantages of stability combined with minimum hazard attendant upon its use. While H_2SO_4-acetic anhydride mixtures must be used shortly after they have been prepared (21, 23), the proposed modified reagent has shown no measurable deterioration during long periods of storage at room temperature. Further, contrary to experience with the mixed Liebermann-Burchard reagent, no violent reactions are occasioned by accidental contamination of the modified reagent with water. When the reagent is added to a mixture of acetic anhydride and acetic acid, no appreciable rise in temperature is observed, nearly all of the heat of reaction of H_2SO_4 with these reagents having been previously expended during preparation of the H_2SO_4-acetic acid reagent. Development of color, therefore, proceeds under essentially isothermal conditions.

Advantages of Deproteinization

Although we have been able to apply the proposed technic to the determination of cholesterol without prior removal of protein, this modification is not recommended. If the precipitated protein is not removed by centrifugation, it will eventually redissolve upon addition of the H_2SO_4-acetic acid reagent, with simultaneous development of the characteristic Liebermann-Burchard color. However, this modification is characterized by lower color intensity and higher serum blanks due to protein-associated pigments. Only about 90 per cent of expected Liebermann-Burchard readings for cholesterol are observed in the presence of serum proteins. A similar finding is available in Fig. 1 of the paper by Pearson, Stern, and McGavack.

Use of Decanted Protein-free Extract Without Measurement of Its Volume

Ten samples of serum were treated in weighed tubes with glacial acetic acid and acetic anhydride according to the described procedure. The volume of residue which remained in each tube after centrifugation and decantation, as determined by weighing and correction for density, averaged 0.23 $\pm$ 0.02 ml. Thus, whereas the addition of 1 ml. of H_2SO_4-acetic acid reagent to the decanted extract reduces the relative concentration of cholesterol to 82.7 per cent (*i.e.*, 4.77/5.77), the standard, which is not decanted, is correspondingly diluted to 83.3 per cent (*i.e.*, 5/6). For accurate results, therefore, a small correction factor, 1.007 (*i.e.*, 0.833/0.827), may be applied in calculation. The average error attendant upon the use of this factor is less than 0.1 per cent. This device serves to simplify operations and to minimize handling and pipetting of a somewhat unpleasant mixture. When tubes of dimensions other than those recommended are employed, a new correction factor should be determined experimentally. For more critical studies, a correction factor may be dispensed with by mixing 4.0 ml. of the protein-free extract with 0.8 ml. of H_2SO_4-acetic acid reagent.

Determination of Free Cholesterol

The determination of free cholesterol is not described in detail, since technics adequate for the isolation of the free sterol are available (19, 22, 24). In our laboratories the ether-washed precipitate of cholesterol digitonide (22) is dissolved in 0.8 ml. of glacial acetic acid at 70°. After the addition of 0.2 ml. of water and 4.0 ml. of acetic anhydride, the technic described for total cholesterol is followed.

Comparative Chromogenicity of Free and Total Cholesterol

It must be emphasized that *all* studies of the Liebermann-Burchard reaction which have demonstrated greater chromogenicity for esterified cholesterol were conducted in chloroform solutions (5, 7, 8, 15, 23, 26, 29). Although it may seem reasonable to suppose that similar differences in chromogenicity would also appear in an acetic anhydride–acetic acid medium, the results of our studies, summarized in Table 1, prove equal chromogenicity. Concordant findings have been reported by others (1, 13, 17). Since a side-by-side comparison of both sets of conditions for color development has apparently not been reported, it seemed desirable, also, to apply the Bloor technic (2) to solutions of free and esterified cholesterol in chloroform. The magnitude of the deviations from equal chromogenicity in chloroform solution, as shown in Table 1, is in essential agreement with the reported findings of others (5, 7, 29). The need for saponification of cholesterol esters is, therefore, determined by the nature of the solvent in which the Liebermann-Burchard reaction is to be performed. When the solvent is chloroform, a saponification step is required; for color development in acetic anhydride–acetic acid medium saponification is superfluous.

Specificity

A direct study of the specificity of the proposed method has not been made, but the work of Abell *et al.* may be cited in this connection. By application of a countercurrent distribution technic, these investigators demonstrated that more than 99 per cent of the material in serum which responded to the Liebermann-Burchard test was actually cholesterol. Excellent agreement with the method of Schoenheimer and Sperry was reported, indicating equal specificity for the latter technic. Table 4 also shows equally good agreement between our proposed method and that of Schoenheimer and Sperry. All three procedures, therefore, appear to be equally specific for cholesterol in serum.

General Considerations

Success in application of the recommended technic is largely dependent upon adequate control of the concentration of water. The same initial volume of water, 0.2 ml., should therefore be present in the standard as well as in the test sample. For this purpose, no significant error is made if the water content of serum is assumed to be 100 per cent. Subsequent accidental inclusion of water is to be scrupulously avoided. Similarly, all stored reagents should be kept in tightly stoppered bottles. The presence

of water produces a change in the proportion of acetic anhydride to acetic acid in the final solution. Since 0.2 ml. of water reacts with 1.05 ml. of acetic anhydride to produce 1.27 ml. of acetic acid, the relative volumes of acetic anhydride to acetic acid are altered from 4.0:0.8 to 2.95:2.07 upon hydrolysis (or dehydration). For best results this final ratio should be maintained constant, because the intensity of color as well as the rate of color development varies with the concentration of acetic anhydride.

It is characteristic of the various modifications of the Liebermann-Burchard reaction that the rate of decomposition of the derivative of cholesterol which is responsible for color is generally quite appreciable in comparison to its rate of development. The existence of a peak, in place of a more suitable level plateau, in the time-absorbence curve necessitates a fixed period of color development prior to absorbence measurement. The method of Schube, which utilizes the presumably more stable "end-product" brown color for measurement has been reported to produce a wide variation in results (23). The degree of stabilization of color which is attained by the present procedure compares favorably with other methods. According to Fig. 1, the greatest variation in absorbence is 1.1 per cent during the 15–25 minute interval following the addition of reagent, and only 0.3 per cent during the 18–22 minute interval. Somewhat better stabilization may be achieved by substituting for the 1:1 reagent an alternate reagent, prepared with 1 volume of concentrated H_2SO_4 and 3 volumes of glacial acetic acid. With this reagent a maximum variation of 0.3 per cent in absorbence was observed during the interval: 45–55 minutes after initiation of color development. This modification would be useful when 20 to 50 specimens are to be analyzed in sequence.

Correction for the blank absorbence of normochromic serums is relatively small. However, inclusion of a serum blank is recommended for improved accuracy. Because the addition of dehydrating reagent occasionally causes turbidity, this reagent is normally omitted from the serum blank. Test samples are not affected by this turbidity, since it disappears readily upon addition of the H_2SO_4-acetic acid reagent. When hyperbilirubinemic serums are analyzed, however, the dehydrating reagent should be added to the serum blank, because H_2SO_4 in the modified color reagent causes a significant alteration in the color of bilirubin. No turbidity has been observed following the addition of dehydrating reagent to hyperbilirubinemic serums.

Some modifications of the basic procedure have been discussed. They

include an extension of the period of color development, if desired, by employing a modified color reagent, prepared with 1 volume of H_2SO_4 and 3 volumes of acetic acid. Also, the correction factor, 1.01, may be eliminated by adding 0.8 ml. of H_2SO_4-acetic acid reagent to 4.0 ml. of serum extract plus 1 drop of dehydrating reagent. Other modifications may readily suggest themselves. When an abnormally high cholesterol value is anticipated, less than 0.2 ml. of serum may be taken for analysis. In this instance, water sufficient to bring the total volume to 0.2 ml. is to be added. Microadaptations may be applied simply by proportional reduction of the standard volumes of all solutions. For example, 0.05 ml. of serum is treated with 0.2 ml. of glacial acetic acid and 1.0 ml. of acetic anhydride in a small tube. After centrifugation, 1.0 ml. of clear supernatant fluid is transferred to a small cuvet, *e.g.*, Coleman No. 6-310; it is then treated with 0.02 ml. of a 5 per cent solution (v/v) of H_2SO_4 in acetic acid, to effect dehydration. Color is developed with 0.2 ml. of H_2SO_4-acetic acid reagent for 20 minutes at 25°. The analysis is completed in the usual manner by measuring absorbences of the sample, a suitable standard, and a serum blank against a reagent blank.

SUMMARY

An accurate yet simple procedure for the determination of total cholesterol, based upon the application of a Liebermann-Burchard color reaction directly in the solvent employed for extraction of cholesterol from serum, has been described. Extraction of cholesterol and removal of protein are accomplished by means of acetic acid and acetic anhydride. Serum water is removed by the acid-catalyzed hydrolysis of acetic anhydride. The Liebermann-Burchard color is then developed with a stable, modified reagent consisting of equal volumes of H_2SO_4 and acetic acid.

Excellent agreement with the technic of Schoenheimer and Sperry is obtained. Equal intensities of color are produced by equivalent concentrations of free and esterified cholesterol. Preliminary saponification of cholesterol esters is therefore not required. Color development may proceed in ordinary room lighting without loss of accuracy.

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Multiple Determinations with Moving-Boundary Electrophoresis

Irwin Oreskes and Harold Corey

MOVING-BOUNDARY ELECTROPHORESIS is a widely used technic for the investigation of colloidal systems. A limitation of this method, however, is the considerable amount of time required for a single experiment. It is particularly time consuming where the accumulation of large amounts of data is necessary. Furthermore, in the majority of instruments only 1 experiment at a time, and usually a total of only 2–3 per day, can be performed.

In a previous publication (1) an electrophoresis cell was described in which two determinations can be performed simultaneously. Using such dual-analysis cells, the authors have developed a procedure for multiple electrophoretic analysis which permits up to 12 such experiments to be performed in an 8-hour day.

APPARATUS AND PROCEDURE

The dual-analysis cell is of a one-piece design and consists of two U-tubes so connected as to constitute a parallel electric circuit whose branches are of equal path length. A separate filling capillary is connected to the base of each U-tube. Generally, the cell is filled in the same manner as the single U-tube type. However, the boundaries in both channels must be formed simultaneously. If the boundaries are formed sequentially, the first one cannot be halted merely by closing off its supply, since the movement of the second boundary further displaces the first.

From the Isaac Albert Research Institute, Department of Physical Chemistry of the Jewish Chronic Disease Hospital, Brooklyn, N. Y.

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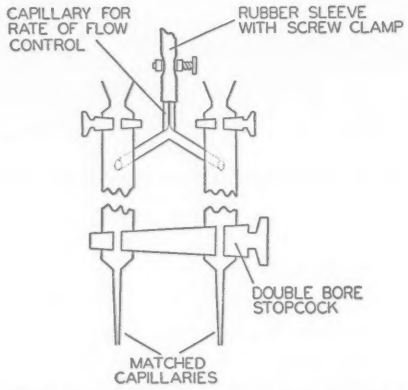

Fig. 1. Filling device for dual-analysis cell.

Filling Device

Simultaneous boundary formation is achieved by the use of a filling device (Fig. 1). This consists of two matched burets that are controlled by one large double-bore stopcock so that they drain at the same rate. The burets are connected near the top by an inverted Y whose vertical limb ends in a fine capillary that acts as a brake on the flow rate. The width between the buret tips and capillaries of the dual-analysis cell are the same, so that for the filling process they are easily connected by short rubber sleeves. This also minimizes the danger of trapping air bubbles. The time involved in filling the dual cell is no longer than that required to fill a single cell. If the viscosities of the two solutions differ greatly the flow rates can be equalized by suitably adjusting the stopcocks on the cell capillaries.

Auxiliary Bath

The technic of multiple analyses, as employed in our laboratory, permits the performance of 4 simultaneous electrophoretic experiments. This is achieved by the use of two dual-analysis cells connected in series. One cell is placed in the water bath of the electrophoresis instrument (Aminco Portable) and the other in an auxiliary water bath (Fig. 2). The temperature of the auxiliary is maintained at 0° by means of ice cubes and mechanical stirring. The ice is confined to one end of the water

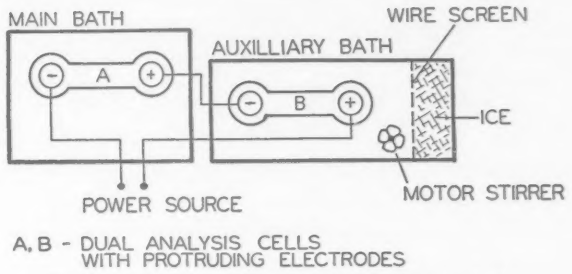

Fig. 2. Schematic arrangement of cells in water baths.

bath by a removable wire mesh gate. The bath is made of copper, 15 x 5 x 10 inches high, and is insulated and reinforced with plywood. Current for the cell in the auxiliary bath is obtained by splicing a wire on to each lead of the power supply. The maximum output of our instrument is 30 ma, and since the cells are connected in series, this will be the current through each one. However, the two channels in each dual cell are internally connected in parallel so the current will divide proportionally to the inverse resistance of each channel. Since these resistances are virtually the same, the current will be equally divided and a maximum of 15 ma can be obtained. This is ample for most experiments. Where the same voltage is required across each cell, rather than the same current, they need only be connected externally in parallel.

When setting up a simultaneous run the cells are filled with buffer on the laboratory bench and electrodes inserted. One cell is placed in the instrument water bath and the boundaries formed and photographed. Then the cell is carefully lifted out and placed in the auxiliary bath, which is already at the correct temperature. The second cell is now placed in the instrument bath and, after filling, the two are connected with the proper current leads. At the end of the experiment the reverse order for photographing the patterns is followed.

RESULTS AND DISCUSSION

The development of an instrumental technic usually follows two paths. One is the refinement of the instrument itself, increasing its analytical or resolving power. The other is the increasing of its productivity in a given time period. The procedure described above permits one worker to increase the output of one apparatus sixfold. Table 1 shows the results of

Table 1. Relative Percentage Concentration of Serum Protein Components

Three Runs Using Four Aliquots Per Run

Exper. no.		Albumin %	mean %	α_1 %	mean %	α_2 %	mean %	β %	mean %	γ %	mean %
1	L	53.8	54.2	7.0	7.2	14.4	14.1	12.6	12.4	12.3	12.1
	R	54.7		7.4		13.7		12.1		12.0	
2	L	54.6	54.2	7.0	7.1	14.0	13.9	12.1	12.3	12.3	12.5
	R	53.9		7.2		13.8		12.5		12.7	
3	L	55.5	54.8	6.8	6.8	13.6	13.7	12.4	12.8	11.7	11.9
	R	54.2		6.7		13.8		13.1		12.1	
4	L	53.3	54:2	7.0	6.5	13.2	13.4	12.6	12.4	14.0	13.6
	R	55.1		6.0		13.6		12.1		13.2	
5	L	54.8	53.9	7.6	7.5	13.7	13.8	12.1	12.5	12.4	12.6
	R	53.0		7.4		13.9		12.8		12.8	
6	L	54.6	53.9	7.7	7.6	12.9	13.3	12.9	12.6	11.8	12.5
	R	53.2		7.5		13.8		12.4		13.2	
Mean		54.2		7.1		13.7		12.5		12.5	
S.D. Corr.		0.79 (single)	0.34 (paired)	0.46 (single)	0.41 (paired)	0.46 (single)	0.29 (paired)	0.34 (single)	0.18 (paired)	0.66 (single)	0.58 (paired)

L, left channel; R, right channel. Odd-numbered experiments done in instrument bath; even ones done in auxiliary bath.

3 runs with pooled human serum, using a pair of dual-analysis cells containing 4 aliquots per run, all performed in a single day. The relative percentage concentration of all components in each experiment is given. It can be seen that the standard deviations of the paired runs is considerably reduced; for example, in the case of albumin by 57 per cent. It is clear that when electrophoretic experiments are done in duplicate, as with the dual cell, considerably greater precision of results can be obtained. The range of values in this series is largely due to variations introduced during tracing and planimetry of the finished patterns, not in the prior experimental steps.

For the past two years the authors have used the auxiliary-bath procedure for single, dual, and moving-section cells in analyzing a wide variety of colloids. It has been very useful for rapidly obtaining statisti-

cally significant protein patterns of pathologic and normal fluids. It is to be emphasized that no detectable disturbances are introduced into the electrophoretic pattern by carefully transferring a cell from one water bath to the other.

SUMMARY

A procedure has been described for the performance of multiple moving-boundary electrophoresis experiments. By connecting two dual-analysis cells in series, 4 experiments at a time and 12 in a working day may be performed.

REFERENCE

1. Corey, H. and Oreskes, I., *Science* **120**, 675 (1954).

The American Board of Clinical Chemistry, Inc., held its annual meeting at the Haddon Hall Hotel in Atlantic City on April 15, 1956. Five clinical chemists, who successfully passed the examination given by the Board last October were certified: W. Knowlton Hall, W. Burkett Mason, Harold Persky, Edwin M. Richardson, and Guilford G. Rudolph. This brings the total number of certified clinical chemists to 246.

A complete *Directory of Certified Clinical Chemists* is available without charge to hospital departments or other laboratories engaging in clinical chemistry. This may be obtained from Dr. William A. Wolff, Bowman Gray School of Medicine, Winston-Salem 7, N. C.

Clinical chemists interested in being certified should also write for the Instructions to Applicants for Certification. In order to be considered for admission to the next regional examinations, which will probably be held during October, 1956, candidates should file their applications immediately.

The Board elected the following officers for the fiscal year July 1, 1956, to June 30, 1957: *President*, Marschelle H. Power, Mayo Clinic, Rochester, Minn.; *Vice-President*, Warren M. Sperry, New York State Psychiatric Institute, New York, N. Y.; and *Secretary-Treasurer*, William A. Wolff, Bowman Gray School of Medicine, Winston-Salem, N. C. During the above fiscal year, the other members of the Board will be: Albert L. Chaney, Joseph W. E. Harrisson, Robert M. Hill, Clarence W. Muehlberger, Arnold E. Osterberg, Joseph I. Routh, and Harry Sobotka. Members elect, whose active membership will begin at the end of the above period, are: R. M. Archibald and Joseph H. Gast.

BOOK REVIEWS

Methods of Biochemical Analysis

DAVID GLICK (Ed.). New York, Interscience Publishers, Inc., 1955, Vol. 2, 470 pp. $9.50.

The second volume of Glick's *Methods of Biochemical Analysis* follows in the steps of the first volume and provides a most useful compendium of analytical methods for the biochemical laboratory. A variety of subjects (listed here in the order of number of articles)—enzymes, proteins, carbohydrates, vitamins, and lipids—are treated in 13 articles. The selection is slightly more concentrated than in Volume 1; the number of pages of text is about 15 per cent smaller. An author's index and a cumulative index have been added.

As was pointed out in the review of Volume 1 (*Clin. Chem.*, **6**, 46 (1954)), this type of publication, reviewing as it does in such active fields as, for example, folic acid, cannot remain up to date. The article on tocopherol appears to be the transciption of a talk, to judge from the intimate way in which the author addresses the reader. Sperry's masterful article on lipide analysis will find immediate practical application in hospital laboratories, but the whole book will be of almost unlimited interest to all clinical chemists. Within a few weeks we obtained useful and time-saving information in such articles as Determination of Serum Glycoproteins and especially on color reactions for sugars. We congratulate the editor and publishers on an excellent volume and are looking forward to its successors.

Mt. Sinai Hospital, N. Y. HARRY SOBOTKA

Official Methods of Analysis of the Association of Official Agricultural Chemists (ed. 8).

WILLIAM HORWITZ (Ed.), H. J. FISHER, A. H. ROBERTSON AND HELEN REYNOLDS. Washington, D. C., Association of Official Agricultural Chemists, 1955, 1008 pp., $12.00

In this eighth edition, the A.O.A.C. presents its methods as revised since the publication of the seventh edition in 1950. All changes in methodology adopted up to and including those made at the Association's annual meeting, October, 1954, are given.

This edition has been expanded by approximately 100 pages, chiefly in the fields of pesticides, flavoring extracts, drugs, microchemical methods, and nutritional adjuncts, including vitamins. A new chapter on spectroscopic methods and a section on hormone drugs have been added, as well as numerous individual methods of analysis in almost every chapter.

For those not familiar with the compilation of these official procedures, the methods are adopted by the A.O.A.C. only after collaborative studies demonstrate that they are reliable, have convenient practical application and give reproducible results in the hands of professional analytical chemists. These standardized procedures are used by governmental regulatory agencies, federal, state, and municipal, and by the regulated industries; in specifications, governmental and private; and by research workers in agriculture. It is a work which is recognized throughout the world.

This book is an excellent reference source for the chemist who is called upon to perform analytic procedures which may not be routine in his laboratory. Besides providing methodology for purely agricultural assays, it provides schemes of analysis for foods, contaminants, drugs, cosmetics and other material of general public use. The chemist in most cases is given a choice of analytic procedures and may choose the one most suitable with available laboratory facilities at his disposal. This is a volume which should be on every laboratory bookshelf.

Metropolitan Hospital, New York, N. Y. H. D. Appleton

ABSTRACTS

Editor: Ellenmae Viergiver. *Contributors:* Joseph S. Annino, Gladys J. Downey, Clyde A. Dubbs, Alex Kaplan, Margaret M. Kaser, Miriam Reiner, Herbert Thompson

The metabolic effects of progesterone in man. R. L. Landau, D. M. Bergenstal, K. Lugibihl, and M. E. Kascht (*University of Chicago, Chicago, Ill.*).

The administration of progesterone in approximately physiologic amounts of 50–100 mg. exerted a mild to moderately intense catabolic influence in men and women. This response was not mediated by the adrenals and there was no decisive evidence that there was thyroid activation. The accelerated protein catabolism appeared to be an effect of progesterone itself. Nitrogen losses were usually greater in patients with adrenal deficiency where the enhanced N excretion was accompanied by an increase in urinary inorganic P, and in 2 cases, by an increase in urinary K. The excretion of these constituents was not increased in non-Addisonians. Creatine retention was found only in several normal subjects.

The same amounts of progesterone induced sharp rises in urinary Na and Cl excretion which were considerably greater in Addisonians treated with corticoids. The loss of Na exceeded the Cl loss. Subjects with functioning adrenals retained Na and Cl in the immediate posttreatment period. In an Addisonian patient, no loss in urinary Na and Cl was induced by progesterone if it was given prior to hormone replacement therapy, but the usual salt loss was observed during corticoid treatment. The posttreatment retention of salt which occurred only in normal subjects suggests an enhanced secretion of salt-retaining corticoids. Such increased adrenal secretory activity could also explain the more limited salt losses in subjects without adrenal effects.—*J. Clin. Endocrinol. and Metabolism* **15**, 1194 (1955). (M. R.)

Thyroid function following hypophysectomy in man. M. C. Li, J. E. Rall, J. P. MacLean, M. B. Lipsett, B. S. Ray, and O. H. Pearson (*Sloan-Kettering Institute, New York, N. Y.*).

Thyroid function has been studied in 28 patients following complete hypophysectomy and in 7 patients following incomplete hypophysectomy. Within 4 weeks after total hypophysectomy, values for serum PBI and thyroidal I^{131} uptake fell to myxedema levels. Clinical hypothyroidism developed within 4 to 8 weeks following total hypophysectomy in 26 of the 28 patients. Serum PBI level and thyroidal I^{131} uptake either fell transiently or did not change in pa-

tients with incomplete hypophysectomy. The development of both laboratory and clinical hypothyroidism appears to be a good index of complete hypophysectomy.—*J. Clin. Endocrinol. and Metabolism* **15,** 1228 (1955). (M. R.)

Acute effects of intravenous hydrocortisone on glucose and insulin tolerances, and levels of serum and urinary inorganic phosphorus. M. Persdy, J. Linsk, M. Isaacs, J. P. Jenkins, M. Rosenbluth, and H. S. Kupperman (*New York University Medical Center, New York, N. Y.*).

The acute effects of the intravenous administration of 17-hydroxycorticosterone on glucose tolerance, insulin tolerance, and the level of inorganic serum P were studied. Infusions of the hydrocortisone were administered alone, with oral glucose, and with intravenous insulin. During hydrocortisone infusion alone, no hyperglycemic effect was noted; during the hydrocortisone-glucose tolerance test there was a consistent decrease in glucose tolerance, but no anti-insulin effect could be shown. Profound decreases in serum P were observed during the hydrocortisone-glucose tolerance tests. Decreased eosinophil counts indicated that active physiologic effects were achieved.

Postulated mechanisms for changes in glucose metabolism are: (1) increased gluconeogenesis; (2) increased peripheral glucose utilization; (3) secretion of a hyperglycemic-glycogenolytic factor from the alpha cells of the pancreas; and (4) inhibition of reflex secretion of insulin under induced alimentary hyperglycemia.—*J. Clin. Endocrinol. and Metabolism* **15,** 1247 (1955). (M. R.)

Determination of methanol in body fluids. F. Stratton (*Medical Examiners Service, Suffolk County, Boston, Mass.*).

The widespread commercial use of methanol and an increase in the occurrence of fatal methanol poisonings led to a study of blood methanol levels by a procedure developed in the author's laboratory. *Procedure:* Place 10 ml. of blood in a 125 ml. distilling flask, add a small fragment of paraffin, connect to a steam generator and condenser and steam distill to give exactly 50 ml. for Fraction I and collect the next 10 ml. as Fraction II to check for complete distillation. Pipet two 10-ml. aliquots of Fraction I and the 10 ml. of Fraction II into separate test tubes and add 1 ml. of H_3PO_4 (85%) and 2 ml. of 3% $KMnO_4$ solution. Mix and let stand 10 minutes to oxidize. Then add 2 ml. of saturated $H_2C_2O_4$ solution and let stand until bleached. For a rapid qualitative test, add to one of the oxidized-bleached aliquots of Fraction I 5 ml. of 1:5 H_2SO_4 solution and 5 ml. of reduced fuchsin and let stand. Methanol gives a blue-to-violet color. For the quantitative procedure, place 100 mg. of chromotropic acid in a 50 ml. beaker. Add 1 ml. of the bleached, oxidized solution and evaporate to dryness on a steam bath. Heat an additional 5 minutes. Add 5 ml. conc. H_2SO_4 and heat on steam plate for 30 min. Cool, pour into a portion of distilled water and make volume to 50 ml. Read at 570 mμ against a 10-ml. water blank which has been similarly treated. Ethanol does not interfere and the color is stable.

In 40 cases of methanol poisoning, 38 of which were fatal, blood methanol levels ranged from 0.01 to 0.52 Gm. per 100 ml.

Reagents required: (1) Reduced fuchsin solution: dissolve 1 Gm. of fuchsin in 1 L. of distilled water, add 50 ml. saturated bisulfite solution and acidify with 1 ml. of conc. H_2SO_4. This solution is allowed to stand until bleached, after which the sediment is removed by filtration through glass wool. (2) Chromotropic acid, (1,8-dihydroxynaphthalene-3,6 disulfonic acid) Matheson Company's P5425 or Eastman Organic Chemical P230.—*Am. J. Clin. Pathol.* **25,** 1071 (1955). (H. E. T.)

A self-standardization method for flame spectrophotometric determination of calcium in biologic materials. C. F. Rothe and L. A. Sapirstein (*College of Medicine, Ohio State University, Columbus, Ohio*).

Measurements were made with a Model DU Beckman spectrophotometer with Model 9200 flame attachment and Model 4300 photomultiplier attachment. In the authors' procedure, an aliquot of the unknown itself is used in compounding the standard. The method is easy, sensitive, and relatively rapid. Because of its self-standardizing characteristics, it is well adapted to biologic materials of widely varying composition.—*Am. J. Clin. Pathol.* **25,** 1076 (1955). (H. E. T.)

A simple R_f calculator for paper strip chromatograms. J. A. Durant (*Portsmouth and Isle of Wight Area Pathological Service, Portsmouth, England*).

Details for the construction of a simple R_f calculator for paper-strip chromatograms are described.—*Am. J. Clin. Pathol.* **25,** 1094 (1955). (H. E. T.)

A simple, rapid procedure for the estimation of albumin and alpha, beta, and gamma globulin in serum. J. C. Aull and W. M. McCord (*Medical College of South Carolina, Charleston, S. C.*).

A simple, rapid method for the determination of albumin, and alpha, beta, and gamma globulin is described which is based upon the measurement of the turbidity produced when serum is mixed with phosphate solutions of specified concentrations. Only 1 ml. of serum is required and one stock phosphate solution which is diluted for use. Turbidity measurements were made with a Coleman Model 11 Spectrophotometer fitted with a special adapter to take 22-mm. test tube cuvets. Results were found to correlate closely with those obtained by the method of Wolfson and associates [*Am. J. Clin. Pathol.* **18,** 723 (1948)].—*J. Lab. Clin. Med.* **46,** 476 (1955). (G. J. D.)

A micromethod for determination of serum calcium. H. E. Harrison and H. C. Harrison (*Johns Hopkins University School of Medicine, Baltimore, Md.*).

A micromethod using 0.2 ml. of serum for the determination of Ca is described. Ca is precipitated as the oxalate at *p*H 4.5 to 5.0 with mechanical agitation to achieve complete precipitation in 30 min. No washing of the precipitate is necessary since excess precipitating reagent and most of the serum Mg is removed by aspiration of the supernatant following centrifugation. The precipitate is then dissolved in disodium dihydrogen ethylenediaminetetraäcetic acid (EDTA) solution in ethanolamine buffer, *p*H 9.5. Using eriochrome black T as indicator, the excess chelating agent is measured by back titration with standard $CaCl_2$ solution in a microburet. The method gives results in close agreement with those obtained with the Clark-Collip modification of the Kramer-Tisdall method. The determination requires 1 to 2 hours for completion.—*J. Lab. Clin. Med.* **46,** 662 (1955). (G. J. D.)

An improved palladium chloride method for the determination of carbon monoxide in blood. T. H. Allen and W. S. Root. (*Columbia University, New York, N. Y.*).

A special apparatus consisting of two 25-ml. Erlenmeyer flasks joined by a ground-glass connection is required. One flask contains the blood sample plus a ferricyanide-saponin solution. The second flask contains solutions of aluminum sulfate and palladium chloride. The flasks are sealed together and rotated for 3 hours. The contents of the second flask are then filtered, treated with KI, and the density of the resultant color is determined at 490 mμ. Results with this method agree with those obtained by a gasometric technic.— *J. Biol. Chem.* **216,** 319 (1955). (J. S. A.)

The determination of total base of urine with an ion exchange resin conditioned as iodate. J. C. Vanatta and R. M. Landers, Jr. (*University of Texas-Southwestern, Dallas, Tex.*).

A method for the determination of total base in serum [*J. Biol. Chem.* **208,** 195 (1954)] has been modified to determine total base in urine. Total base and total fixed base (total base plus ammonia) may be determined. The preliminary removal of phosphates by precipitation with ferric alum is necessary before determining total fixed base. The average error as determined by replicate analyses is less than 2%.—*J. Biol. Chem.* **216,** 351 (1955). (J. S. A.)

A flame photometric method for the determination of plasma magnesium after hydroxyquinoline precipitation. S. Davis (*Veterans Administration Hospital, Indianapolis, Ind.*).

A new method is described for separating Mg from other substances which interfere in the determination of Mg by flame photometry. The blood sample is added to oxalate to precipitate Ca. After centrifugation the Ca-free plasma is treated with trichloroacetic acid to precipitate proteins. The filtrate is treated with hydroxyquinoline and incubated to precipitate Mg. The precipitate is

separated and washed with the aid of a special filter stick. Na and K are effectively removed in the washing process. The dried precipitate is redissolved in an acetone-acetic acid mixture and the Mg concentration is determined using the Beckman DU oxyhydrogen flame photometer. The results obtained by this procedure agree with those obtained by the titan yellow method.—*J. Biol. Chem.* **216,** 643 (1955). (J. S. A.)

The mechanism of satiety: Effect of glucagon on gastric hunger contractions in man. A. J. Stunkard, T. B. Van Itallie, and B. B. Reis (*New York Hospital and St. Luke's Hospital, New York, N. Y.*).

The subjects were 7 healthy adults in the postabsorptive state. Gastric hunger contractions were recorded on a kymograph by means of a water manometer connected by a Levin tube to an inflated balloon in the stomach. Capillary and venous blood samples were analyzed for glucose by the Nelson-Somogyi method. On 16 occasions intravenous injection of glucagon (hyperglycemic glycogenolytic factor) in a single 2-mg. dose, in repeated small doses, or by continuous infusion, caused abolition of gastric hunger contractions within 3 minutes accompanied by disappearance of the sensation of hunger. At the same time there was a marked increase in the blood sugar level with an increase in the capillary-venous difference. The return of the blood sugar to normal was associated with a renewal of hunger contractions.—*Proc. Soc. Exp. Biol. Med.* **89,** 258 (1955). (M. M. K.)

Clot density determination of fibrinogen in experimental myocardial infarction. B. W. Volk, S. Losner, and P. Crastnopol (*Jewish Chronic Disease Hospital, Brooklyn, N. Y.*).

A rapid method [Losner *et al. J. Lab. Clin. Med.* **38,** 28 (1951)] was used for the estimation of fibrinogen. Fifty specimens of citrated dog plasma were diluted with saline to 80%, 60%, 40%, and 20%. The clot density differences were found to be linearly related to the concentrations of fibrinogen and were the same as for samples of human plasma similarly treated. Twenty dogs were subjected to high coronary artery ligation, 20 to low coronary artery ligation, and 10 to sham operation. The maximum fibrinogen concentration in the first group of animals was 588 mg. per 100 ml., 516 mg. in the second group, and 408 mg. in the third. The duration of the elevation was longest for the first group. These results agree with a clinical impression that the maximum fibrinogen concentration parallels the extent of myocardial infarction.—*Proc. Soc. Exp. Biol. Med.* **89,** 277 (1955). (M. M. K.)

Urinary levels of mucoprotein components in normal male subjects. D. Hamerman and F. T. Hatch (*Fitzsimons Army Hospital, Denver, Colo.*).

Data are presented for analyses of the high molecular weight material obtained by dialysis from the urine of 8 normal male subjects. The mean values in milli-

grams per 24 hours were as follows: nitrogen, 35.89; fucose, 6.18; total hexosamine, 28.90; total hexose, 38.43; and hexuronic acid, 5.81. With phosphotungstic acid precipitation the values for total hexosamine and hexose were 14.04 and 14.14 mg. per 24 hours, respectively.—*Proc. Soc. Exp. Biol. Med.* **89,** 279 (1955).

(M. M. K.)

Determination, recovery, identification and renal clearance of conjugated adrenal corticoids in human peripheral blood. A. M. Bongiovanni and W. R. Eberlein (*University of Pennsylvania, Philadelphia, Pa.*).

Freshly drawn plasma was added to 2.5 volumes of 95% ethanol, and the precipitate was removed by filtration or centrifugation. After the ethanolic solution had been evaporated to dryness with air at 45°, a volume of water equal to the original volume of plasma was added to the residue, and the mixture was extracted 3 times with ⅔ volume of distilled methylene chloride. The extract was evaporated to dryness and the residue was chromatographed on florosil according to Nelson and Samuels [*J. Clin. Endocrinol. and Metabolism* **12,** 519 (1952)] to remove free corticosteroids. The aqueous phase was incubated with acetate buffer (0.1M, *p*H 4.5) and 300 to 500 units of beta-glucuronidase per ml. at 37° for 48 hours. It was then extracted with methylene chloride and chromatographed on florosil as above. To each residue was added 0.25 ml. of absolute alcohol and 0.75 ml. of Porter-Silber reagent (65 mg. phenylhydrazine hydrochloride in 100 ml. of 62% v/v H_2SO_4. After being allowed to stand in the dark at room temperature for 16 hours, the chromogens were read in the Beckman DU spectrophotometer at 370, 410, and 450 mμ. Compounds E, F, and S and their reduced tetrahydro derivatives are equally chromogenic. Data are presented to show that satisfactory reproducibility and recoveries are obtained with tetrahydro-E.

Blood levels of conjugated corticosteroids in excess of 3 mg. per 100 ml. of plasma associated with only a small increase in the free fraction were found in a human subject after the oral administration of large doses of tetrahydro-E (free alcohol). The material isolated from blood was identified as tetrahydro-E with a second component, possibly the 20-reduced derivative. The free corticosteroids were cleared at an average rate of 11.2% and the conjugated 71.0%, as compared to creatinine.—*Proc. Soc. Exp. Biol. Med.* **89,** 281 (1955).

(M. M. K.)

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2. Giordano, A. S., and Winstead, M.: Am. J. Clin. Path. *23*:610, 1953.
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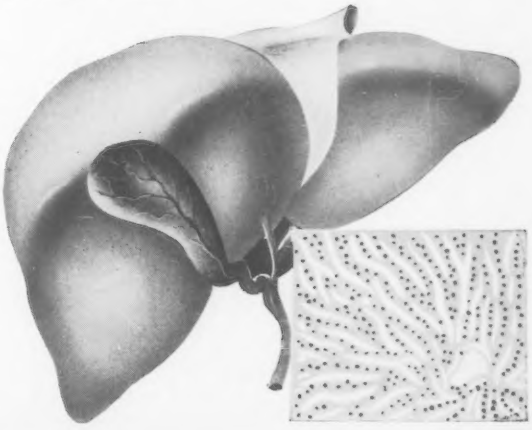

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